

The role of prostaglandins in *Vibrio cholerae* and *Escherichia coli* induced diarrhoea.

by

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DEDICATION

This Thesis is dedicated to
my parents for their continuous
encouragement and support
throughout the course of my
Ph.D.

SUMMARY

Considerable circumstantial evidence suggested that prostaglandins or prostaglandin-like substances might be responsible for the diarrhoea associated with *V. cholerae* and heat-stable *E. coli* enterotoxins. The role of prostaglandins in the secretion of fluid and electrolytes initiated by these toxins was therefore investigated.

Cholera toxin was prepared from *Vibrio cholerae* strain 12r (Ogawa). The prostaglandin content of crude cholera toxin (Wyeth lot 002 toxin) and crude *E. coli* strain B44 heat-stable enterotoxin was determined. Neither contained sufficient prostaglandin-like activity to account for their secretory stimulant properties.

Prostaglandins of the E and A series were released from rabbit intestine by cholera enterotoxin but it was demonstrated by further experiment that this release was associated with tissue trauma and loop distension. Indomethacin administered systemically reduced the prostaglandin release markedly but did not cause a significant reduction in fluid output by the intestine. In in vitro studies it was demonstrated that purified cholera toxin did not modify the synthesis of PGE and PGF compounds by the small intestine.

In cats the effect of prostaglandin synthesis inhibitors on fluid output by the lower and upper small intestine was investigated. Neither systemic nor local application of indomethacin nor systemic administration of aspirin prevented the secretion of fluid by the intestinal mucosa following intra-luminal challenge with cholera enterotoxin. Prostaglandin release was modified by these drugs. The plasma levels of indomethacin achieved by systemic therapy were monitored.

It was shown that levels of indomethacin attained in vivo were sufficient to cause significant reduction in prostaglandin biosynthesis by cat terminal ileum tissue in vitro.

The prostaglandin content of washings from E. coli ^{toxin.} infected piglets and control healthy animals was assessed. It was found that both healthy and infected material contained prostaglandin E and F-like activity. There was no significant difference in prostaglandin content between the two groups of samples.

The ability of E. coli toxin infected intestine and healthy intestinal tissue to synthesize prostaglandin-like compounds was investigated. PGE₂-like and PGF_{2α} activity was detected. The presence of PGF_{2α} was confirmed by combined gas chromatography-mass spectrometry.

The effect of intraluminal administration of PGE₁ and PGA₁ on net ^{water} ~~ion~~ and electrolyte movement across the intestinal mucosa of piglets was studied. The changes in these parameters wrought by the prostaglandins were compared to those initiated by heat-stable Escherichia coli toxin.

Intra-arterial infusion of PGE₁, PGA₁ and PGF_{2α} into the superior mesenteric artery of dogs had been demonstrated to initiate fluid and electrolyte secretion by canine jejunum similar to that observed following cholera and Escherichia coli toxin administration. The ability of intra-arterially administered prostaglandins to mimic the secretory activity of E. coli toxin in calf intestine was investigated.

No evidence was found to implicate prostaglandins or prostaglandin-like compounds in the fluid and electrolyte losses associated with either V. cholerae or E. coli enterotoxins.

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Historical Introduction

Until the early 19th century cholera was a disease confined mainly to India and the neighbouring countries of the East. With the expansion of commerce and the opening up of new trading routes to the East, the occurrence of cholera in other countries was inevitable and in the period from 1817 to 1923 six pandemics were to occur. In 1817 the Bengal outbreak spread rapidly to China, and the disease reached Burma, Thailand and Indonesia two years later. By 1822 Japan, Malaya and the Phillipines had all experienced the effects of the vibrio. It was transmitted to Arabia by the British expeditionary force which was sent from India in 1821 and soon the countries on the Southern Mediterranean coast, Zanzibar and East Africa were infected. The first reports of a westward spread of the disease was in the pandemics which scourged the world between 1826 and 1837. Europe experienced its initial attack in 1829 and both England and Scotland were ravaged by cholera in 1831. It was only after the appearance of the disease in the western hemisphere that any real interest in the scientific aspects of the illness was taken. Sporadic outbreaks of the disease have been reported in Europe in the past decade and with these new incidents fresh interest has been aroused into the mechanism of action of *V. cholerae*.

As early as 1831, it was known that the treatment of cholera necessitated fluid and electrolyte replacement (O'Shaughnessy, 1831) and by 1837 Snow had postulated that the rice-water stool was the effect of hypersecretion in the small intestine due to the toxic substances which acted as potent irritants in the gastro-intestinal tract (Snow, 1854). It was not for some

thirty to forty years that much headway was made, until Koch in 1884 showed that the outbreak of cholera in Alexandria, Egypt, during the previous year was attributable to the comma bacterium. Koch postulated that the disease was not due to the action of the vibrio itself but resulted from the effects of toxic substances elaborated by the organism. He also stated that the disease processes were confined to the small intestine. Evidence to support the participation of toxins came from the work of Nicata and Rietch (Nicata & Rietch, 1884) who showed that week-old cultures of the vibrio caused lethal poisoning when injected intravenously in dogs, a property not shared by fresh cultures of the organism. In 1886 Cantoni found endotoxic activity associated with heat killed cultures of *V. cholerae*. These reports were confirmed in 1892 by Pfeiffer. Pfeiffer demonstrated that young, aerobically incubated cultures of vibrio contained a toxic substance which was resistant to the effects of heat, chloroform and thymol, all of which resulted in the death of the bacteria. Furthermore intraperitoneal injection of the cholera toxin in guinea-pigs caused progressive hypothermia and was lethal in large doses. Lesser doses caused severe incapacitation but the animals generally recovered within twenty-four hours. It should be noted however that these early reports are probably describing the effects and properties of the endo rather than the enterotoxic moieties associated with *V. cholerae*.

Advances in the mechanism of action of the vibrio were retarded at this time due to the influence of Virchow. At post mortem examination Virchow (Virchow, 1879) observed that denudation of the intestinal epithelia had occurred in

specimens from deceased cholera patients. Thus in 1879 he proposed that this loss of epithelial integrity was the cause of the massive diarrhoea experienced by the choleraic patient. Not even the evidence presented by Cohnheim in 1882 was able to refute the claims made by Virchow. Cohnheim (Cohnheim, 1882) asserted that the denudation was a post-mortem artefact and that, as the rice water stool contained so little proteinaceous material, it was due to hyperactivity of normal secretory mechanisms in the intestine, as had been suggested by Snow some years earlier. The presence of relatively high concentrations of amylase in the stools was further evidence in favour of Cohnheim's hypothesis. Even the fresh evidence put forward by Goodpasture in 1923 (Goodpasture, 1923) was insufficient to deter many members of the medical profession from continuing to give credence to the views of Virchow for some further forty years.

During the next half century interest was focused on the nature of the toxic substances produced by *V. cholerae*, and in particular in endotoxic moieties. At this time bacterial diseases were believed to owe their symptoms to endo-rather than exotoxins. Kolle and Schurman (1912) and Kolle and Frigge (1928) had attested that the choleraic syndrome was produced by exotoxins elaborated by the growing organisms. Wilson and Miles stated catagorically^e that no true exotoxins were synthesized by the vibrio but that endo-toxins were released on autolysis or by the action of host cells on the bacteria, suggesting that these bacteria were relatively easily lysed being similar to microcci^{co} in this respect (Wilson & Miles, 1946). Earlier work by Boivin and

Mesrobianu in 1935 and 1936 had shown that *V. cholerae* did produce endotoxin. These workers were able to extract glycolipid endotoxic material from the organisms, a result confirmed by Bernard and Gallut in 1943 and by Burrows in the following year (Burrows, 1944). Gallut working together with Graber demonstrated that *V. cholerae* produced at least two toxic substances (Gallut & Graber, 1943, 1947). Banerjee (Banerjee, 1942) was able to isolate a dialyzable toxin from vibrio cultures using dialysis techniques. Freter confirmed the synthesis of endotoxin in 1953 and later characterized the differences between the endo- and exotoxic material in a more intensive investigation (Freter, 1956). The situation became even more complicated by the early 1960s when two further toxic moieties were described. Huber and Phillips showed that a sodium pump inhibitor was present in cholera stools during the epidemic in Bangkok in 1960 (Huber & Phillips, 1962) and later a capillary permeability factor was isolated by Basu, Mallick and Ganguli and by Craig (Basu, Mallick & Ganguli, 1964; Craig 1965a). Thus at least four toxic substances were implicated in the choleraic syndrome.

In the late 1960s it became apparent that the problem was in fact much simpler than had once been imagined. Exotoxins produced by *V. cholerae* were capable of reproducing the effects of vibrial infection in various experimental models and in human subjects (see appropriate sections of Introduction) and thus the question to be answered was how did the enterotoxic exotoxin stimulate the fluid loss in cholera and how could this process be interrupted.

The work of Ling (1965) and Finkelstein (Finkelstein, Sobocinski,

Atthasampunna, Charunmethee, 1966a) excluded the sodium pump inhibitor as a causative agent of fluid production. The participation of endotoxins was also excluded by the ability of cell free fractions and supernatant from non lysed cell cultures to mimic the effects of bacterial infection (see Latif, 1966, Sheahan, 1972) and the inability of endotoxic material isolated from choleraic stools to reproduce the syndrome in rabbits (Finkelstein, Mukherjee & Rudra, 1963).

Experimental Models

(a) Rabbit Ligated Loop Models.

The first experimental model used to study *V. cholerae* - induced diarrhoea was that of Metchnikoff. In 1892, Metchnikoff (Metchnikoff, 1892) produced the symptoms of cholera in infant rabbits by allowing them to suckle from maternal teats smeared with *V. cholerae* organisms. Because great emphasis was laid on showing the problem of endotoxin versus exotoxin production, animal studies on the intestinal aspects of cholera tended to be neglected somewhat. In 1915 however, Violle and Grendiropoulou developed the ligated intestinal loop model. Using a 10 cm length of upper small intestine they were able to demonstrate fluid production and denudation of mucosal epithelium (Viole and Grendiropoulou, 1915). The infant rabbit model was revised in 1955 by Dutta and Habbu who used intra-intestinal inoculation with *V. cholerae* cultures and obtained reproducible diarrhoea. Later intragastric administration of cell-free filtrate (Dutta, Panse, Kulkarni, 1959), or cell-free ultrasonic lysate (Oza & Dutta, 1963) to infant rabbits was found to give similar results. These results were confirmed by Finkelstein and his associates (Finkelstein, Norris & Dutta, 1964). In parallel

with the development of the infant rabbit model, was the re-introduction of the ligated loop model in adult rabbits (De & Chatterjee, 1953). The model has been used to study the effects of vibrio cultures (De, 1959; De, Ghose & Sen 1960; Formal, Kundel, Schneider, Kunev, Sprinz, 1961). Namioko, Urushido and Sakazaki (1958) and Taylor and his associates (Taylor, Maltby and Payne, 1958) used the loop model to establish enteropathogenicity in a range of *V. cholerae* strains. McNaught and Roberts also used the model but reported that false positive responses could be elicited (McNaught & Roberts, 1958). De and Ghose found that such responses were only obtained when the volume injected exceeded 1 ml/10 cm length of gut (De & Ghose, 1959). The model was modified by Kasuga and his collaborators (Kasuga, Goda, Ushiba, Ujiie & Kishimoto, 1963) and by Hattore's group (Hattore, Misawa, Igarashi & Sugiya, 1965). These groups washed the loops with saline to remove debris and mucin. Longer loops were used by Leitch (1965) and Schafer and Lewis (1965) but even they were unable to avoid false positive results.

The suitability of other animal species has been investigated. Monkeys and goats have both been used but there appears to be no advantage over rabbit models (Fresh, Versage & Reyes, 1964). Rat loops are not susceptible (De, 1961). The guinea-pig has been reported to be a more sensitive preparation (Nikonov, Yevsenyeva, Bibikova & Bichul, 1958).

Further sophistication of the loop technique has led to the introduction of Thiry-Vella loops in dogs (Sack & Carpenter, 1969).

(b) Other Models

Although a certain amount of information is provided by

animal studies, changes in ion and water movement are perhaps more easily studied in in vitro systems. Isolated strips of guinea-pig and rabbit small intestine were used by Burrows, Wagner & Mather in 1944 to investigate the effects of cholera toxin on fluid movement. An increase in the flow of Ringer-Locke solution was induced by *V. cholerae* cells and could be abolished by agglutinating these cells with antisera. Isolated ileal segment models have been employed to measure the magnitude and nature of electrolyte and water fluxes in cholera (Field, Fromm, Wallace & Greenough, 1969; Al Awqati, Cameron, Field, Greenough, 1970a). Studies have been carried out principally on human and rabbit ileum.

Greater confidence has been placed in experimental models since Benyati reported the ability of cell-free material to produce cholera in human subjects. (Benyati, 1966).

Preparation of Cholera Toxin

There are two characteristic features associated with almost every toxin yet found: firstly, extreme potency - a minute quantity of a pure toxin preparation is required to produce the pathological effect associated with the toxin's presence; secondly, specificity of site of action - cholera toxin shows remarkable specificity for the gastro-intestinal tract. Introduction of the toxin at other sites does not lead to the massive fluid production associated with cholera.

Vibrio cholerae produces several toxic substances:

(1) a heat-labile, enterotoxin, which is non-dialyzable, occurs intracellularly, diffuses into the culture medium, is not a cell wall component and is responsible for the intestinal electrolyte and water movement both in vitro and in vivo. It is also

responsible for increased capillary permeability in the skin and cytotoxic effects on cell culture (for references see Reviews of Craig, 1971; Burrows, 1968 and Monograph of Sheahan, 1972). This toxin is also capable of stimulating adenyl cyclase activity in a variety of tissues (see appropriate subsection of introduction for references).

(2) A heat-stable, non-dialyzable factor seen in liquid cultures after autolysis of the cells has occurred and contains mouse lethal factor. This is probably an endotoxin (Craig, 1970; Burrows, 1944).

(3) Heat-stable, dialyzable factors which inhibit sodium transport in anuren epithelia, and para-amino hippuric acid uptake by kidney tissues in vitro (Burrows, 1968; Sheahan, 1972).

This study is concerned only with the enterotoxin produced by *V. cholerae*.

Most of the classical and El Tor strains of *V. cholerae* are of low toxicity but *V. cholerae* 569B has been brought to high virulence by serial passage in animals (Dutta and Habbu, 1955). This strain grows with relative ease on a simple salt-containing medium. Growth conditions leading to optimum toxin production vary from strain to strain, but appropriate adjustment of salt concentration, aeration, pH and temperature will generally lead to toxin manufacture (Craig, 1971), (for references see Monograph of Sheahan, 1972). The toxins obtained from the various strains appear to be antigenically identical, and behave identically on agar gel double diffusion precipitation testing (Finkelstein, Sobocinski, Atthasampunna, Charunmethee, 1966b). Enterotoxin is detectable in the medium during the log phase of growth and reaches a maximum at the end of this phase and the beginning of

the stationary phase. (Richardson, 1969, Latif, 1966).

Richardson demonstrated that lysing of the cells prior to entry into the exponential growth phase inhibited toxin production completely (Richardson, 1969) and thus it seems as if the log phase is of critical importance in the toxin cycle. The functions of these toxins is obscure. It does not appear to be of metabolic consequence for the bacteria. Richardson (1969) has suggested that it may be a non-functional protein.

Purification of Cholera Toxin

Most assays in experimental models have been carried out using relatively impure forms of toxin. Purification is a complex multi-step procedure requiring ammonium sulphate precipitation, DEAE cellulose chromatography and gel filtration (Finkelstein and Lo Spalluto, 1969), or ultrafiltration through various membranes and gel filtration through Sephadex-G75 (Finkelstein and Lo Spalluto, 1972). This latter technique gives better yields and has been used to prepare crystalline toxin and naturally occurring toxoid (Finkelstein and Lo Spalluto, 1972). Other modifications which have been introduced to provide high yields of relatively pure, concentrated toxin, include an initial adsorption on to aluminium hydroxide (Spyrides and Feeley, 1970) but further chromatography has to be used to increase the purity of the end-product (Finkelstein, Fujita and Lo Spalluto, 1971a, Finkelstein, Peterson & Lo Spalluto, 1971b). Richardson, Evans & Feeley, (1970) using a combination of dialysis, dextran sulphate precipitation and elution, ammonium sulphate precipitation, gel filtration and ion exchange chromatography obtained a pure toxin from the activity point of view, but this preparation was found to be contaminated with significant amounts of somatic antigen and

to stimulate the production of vibrocidal antibodies. Furthermore a second band was found on immuno-electrophoresis of the protein material.

Pure crystalline and non-crystalline forms of toxin are not generally available for investigative work in experimental models, thus caution must be exercised in analysing results and interpreting differences obtained with toxins from different sources.

Properties of Cholera Toxin and Toxoid

Cholera toxin is not a stable bacterial product and even with low temperature storage is found to lose its activity over a period of months. At room temperature the process is very much faster (Finkelstein, 1972, Van Heyningen, personal communication). The deactivation mechanism is thought to represent the spontaneous decomposition of toxin into its two subunits, the A fragment of molecular weight 27,000 and the B fragment of approximately 56,000 molecular weight. The B fragment is antigenically identical to the toxoid obtained in purification procedures (Finkelstein and Lo Spalluto, 1969, 1970, 1972).

Table 1 summarises the properties of cholera toxin and the naturally occurring toxoid (modified from Finkelstein 1972).

Property	Cholera Toxin	Cholera Toxoid
Cholera-producing dose infant rabbit model	0.25 - 0.5 μg	100 μg
Skin-reactive dose adult rabbit	$0.6 \times 10^{-4} - 0.1 \times 10^{-2} \mu\text{g}$	0.1 - 0.2 μg
Ileal-loop reactive dose - adult rabbit	0.03 - 0.2 μg	100 μg
Fat cell lipase	0.014 μg	Inactive
Intravenous Lethal Dose Mice	10.0 μg	Inactive
Molecular weight (sedimentation)	84,000	58,000
Number of subunits	6	4
Subunit molecular weight	14,000	14,000
Hexose and Lipid Content	< 1%	< 1%

Table 1. The properties of cholera toxin and its naturally occurring toxoid. The table is modified from that of Finkelstein, 1972.

Site of Action of Cholera Toxin

Cholera toxin has a purely local effect on the gastrointestinal tract. In order to produce the clinical symptoms of cholera, the toxin must come in contact with the luminal side of epithelial cells of the intestine. Injection into the wall can produce an intense inflammatory response but cannot elicit fluid accumulation in the gut lumen (Sheahan and Sprinz, 1971). Similarly serosal application of toxin is ineffectual (Field, Fromm, Wallace & Greenough, 1969). Human studies (Banwell, Pierce, Mitra, Brigham, et al, 1970), and canine Thiry Vella Loop model experiments (Carpenter, Sack, Feeley & Steinberg, 1968) indicate that the whole of the small intestine is susceptible to attack by the toxin. Evidence from experiments in neonate and adult rats by Bayless and his co-workers (Bayless, Luebbbers & Elliott, 1971) indicate that the intestinal crypts may be the precise location of toxin receptors. Neonatal rats which do not have mature crypts are not sensitive to cholera toxin. Peterson, Lo Spalluto & Finkelstein (1972) localized the toxin to the exterior of the epithelial surface: They were unable to demonstrate penetration of the mucosa by the toxin using immunofluorescent and autoradiographic techniques. Elliott and his colleagues (Elliott, Carpenter, Sack and Yardley, 1970) found toxin in close association with the fuzzy coat of the brush border in biopsy studies in dogs during experimentally induced cholera. Again no penetration of mucosal tissue or crypt lumen was observed. Thus the choleraic syndrome presents one with a peculiar problem - how does the toxin cause the massive fluid loss? Evidence has been presented very recently by Cuatrecasas in a series of papers (Cuatrecasas, 1973, a, b, c, d) suggesting that interaction

with a ganglioside component of the epithelial cell wall may be the initial reaction which eventually leads to the pathological manifestations of the disease. This work is supported by similar studies of van Heyningen, and his collaborators, (van Heyningen, WE, Carpenter, Pierce & Greenough, 1971) and by van Heyningen using the ganglioside G_{m1} (van Heyningen, S., 1974). Leitch and Glinkson (1972) observed a decrease in the phospholipid, sialic acid, and cholesterol levels in purified brush border preparations on exposure to cholera toxin. The ratio of phospholipid (P.L.) to cholesterol (Cholesterol) was markedly altered - the ratio decreased from 1:4 (PL: Cholesterol) to 1:2.

Mechanism of Action of Cholera Toxin

The initial step in producing diarrhoea appears to involve an interaction between the 56,000 molecular weight component of cholera toxin and some ganglioside component of the cell membrane (van Heyningen, 1974 and personal communication). The smaller subunit, being essential to produce the toxic effects once the binding step has been effected, has apparently no role in toxin attachment to the membrane.

Several mechanisms have been postulated for the production of fluid.

- (1) increased vascular capillary permeability
- (2) decreased absorption
- (3) Hypersecretion - active secretion of electrolytes and water
- (4) a combination of (2) and (3).

(1) Increased vascular permeability

Evidence both in favour and against the vascular permeability theory has been found. In 1953 De and Chatterjee (De & Chatterjee

1953) noted that fluid which had accumulated in the ligated intestinal loop in rabbits following inoculation with *V. cholerae* culture was rich in protein and that Evans Blue dye could be detected in the fluid following the intravenous administration of this plasma marker. Liebermeister stated that it was not unusual to find variable numbers of red blood cells in cholera stools giving the fluid a mucosanguinous or meat-broth like appearance (Liebermeister, cited in Pollitzer, 1957). Keusch and his co-workers (Keusch, Atthasampunna & Finkelstein, 1967) found increased permeability to iron-dextran complexes in mucosal capillaries. In ultrastructural and anatomical studies (Fresh et al, 1964; Dalldorf, Keusch & Livingstone, 1969; Merrill and Sprinz 1966) dilatation of capillaries was found. Elliott et al, 1970 and Norris and Mayo (1965) were unable to confirm these results. Love (1969) found the pore size of ileal loop mucosa had doubled. Circumstantial evidence also supports this theory - the time course of cholera-mediated fluid loss is very similar to that of permeability changes in inflammation and thermal injury (Formal et al, 1961, Schafer & Lewis, 1965, Wilhelm 1971, Leme, Hamamura, Leite, Rocha e Silva, 1973). No increase in permeability could be detected by Gordon (Gordon, 1969), nor by Rhode and Chen (Rhode & Chen, 1972), and Hakim and Lifson concluded that cholera toxin operated by a mechanism not involved with increases in hydrostatic pressure in the interstitial tissue of the mucosa (Hakim & Lifson, 1969). Protein loss is minimal (Weaver, Johnson & Phillips, 1948) and the ion concentrations in the diarrhoeic fluid do not reflect those of plasma exactly (Carpenter, Barua, Sack, Wallace, Mitra et al, 1966). Cholera toxin does cause an increase in vascular permeability in skin in guinea-pigs

(Kennedy & Richardson, 1972) and in rabbits (Craig, 1965a, Latif, 1966). Injection of the toxin into the wall of the intestine results in a marked inflammatory response (Sheahan & Sprinz, 1971). Alteration in mucosal blood flow does not alter fluid secretion in cholera (Norris & Summer, 1971; Carpenter, Greenough, Sack, 1969a).

(2) Impaired Absorption

Absorptive processes remain intact in cholera - neither glucose absorption nor the lumen to plasma flux of sodium appear to be reduced (Love, 1969; Banwell et al, 1968; Leitch et al, 1968; Grady, Madoff, Duhamel, Moore & Chalmers, 1967; Hirschorn, Kinnie, Sacher, Northrup et al, 1968; Iber, McGonagle, Serebro, Luebbers et al, 1968; Carpenter et al, 1968). Anatomical and ultrastructural studies indicate that the integrity of the gut epithelium is maintained (Gangarosa, Beisel, Benyajati, Sprinz & Piyparatn, 1960). Serebro et al showed that the absorption of glucose was independent of the net ion and fluid movements due to cholera toxin. Enhanced sodium absorption still occurs in the choleraic animal and man, as it does in the healthy controls (Hirschorn et al, 1968; Serebro et al, 1968).

There is a factor in crude preparations and in cell-free culture fluid which is capable of inhibiting sodium uptake by the intestine but Burrows has shown that this substance can be separated from the enterotoxin on the basis of the heat lability and non-dializability (Burrows, 1968). This sodium pump inhibitor is a mucinase enzyme. It is not known whether it is important in cholera in human patients but it does not appear to be essential in experimental models (Sheahan, 1972).

It would therefore seem as if the theory postulated by Fuhrman and Fuhrman and supported by other groups can be discounted and that choleraic diarrhoea is not the result of impaired absorptive mechanisms in the intestine (Fuhrman & Fuhrman, 1959).

Active Secretion

Stimulation of active secretory processes seems to be the most likely mechanism for producing fluid loss in cholera. Water movement occurs against an osmotic gradient and must therefore be coupled to the transport of electrolytes. The coupling has to occur in a restricted space so that an appropriate osmotic environment can be created. Field has proposed that the crypt regions of the intestine are suitable sites (Field, 1971a). Supportive evidence comes from the observation that cycloheximide, which in the intestine is selectively cytotoxic to crypt cells (Verbin & Farber, 1967), interferes with secretory mechanisms and inhibits the effect of cholera toxin while leaving absorptive processes intact. (Serebro, Iber, Yardley, Hendrix, 1969; Grayer, Serebro, Iber & Hendrix, 1970; Harper, Grayer, Yardley & Hendrix, 1970). Moritz, Iber and Moore showed that cycloheximide caused a reduction in sodium and bicarbonate ion secretion and abolished that of chloride ions. Water movement was also abolished (Moritz, Iber & Moore, 1971).

Field, (Field et al, 1969) found that cholera toxin stimulated chloride ion secretion, increased short circuit current and raised the potential difference across membranes in vitro. Leitch and his colleagues found that in vivo cholera toxin not only produced chloride ion secretion but also enhanced bicarbonate

ion efflux (Leitch, Iwert, Burrows, 1966). Al Awqati's group confirmed the results obtained by Field in 1969 (Al Awqati, Cameron, Field, Greenough, 1970a). Bicarbonate ion flux probably accounts for the residual ion flux that is not accounted for by the movement of chloride and sodium ions in control tissues.

In vivo bicarbonate transport appears to be of greater importance than that of chloride ions (Banwell et al, 1970, Leitch & Burrows, 1967, Leitch et al 1966, Moore, Bieberdorf, & Morawski, 1971). Field and his co-workers were able to inhibit the effect of cholera toxin in rabbit ileum preparations in vitro by replacing both the bicarbonate and chloride ions in the Ringer solution with sulphate ions. If only one was removed or if the sodium was omitted and choline substituted for it, then the rise in short circuit current due to cholera toxin was reduced. (Field, Plotkin & Silen 1968). Glucose-independent transport of sodium is inhibited by the toxin while the glucose-dependent pathway is unaffected. Glucose perfusion of the intestine in animals and human patients is able to increase sodium and water transport to such an extent that the effect of cholera toxin is abolished (Serebro, Bayless, Hendrix, Iber, McGonagle, 1968).

Pharmacological Modification of Choleraic Diarrhoea

Therapy in cholera has proved to be a difficult task despite the simplicity of the treatment required to ensure recovery in the majority of patients. Rehydration is essential but the countries ravaged by cholera generally have inadequate hospital facilities and trained staff to carry out this procedure. The administration of antibiotics is of limited use as the

choleraic syndrome is self limiting - the massive diarrhoea serving to purge the intestinal tract of the causative agent (Banwell and Sherr, 1973). Prophylactic vaccination is difficult to execute on the scale required and as protection is of limited duration, affords little hope, as yet, as a means of controlling the disease. (Sheahan, 1972).

Attempts to interfere with fluid production in experimental models of cholera have had mixed success. Ethacrynic acid has been found to inhibit the effects of cholera toxin in in vitro models (Al Awqati, Greenough & Carpenter, 1969). Cycloheximide prevents fluid secretion in rabbit intestine when administered prior to toxin challenge (Serebro, Iber, Yardley & Hendrix, 1969; Moritz, Iber, Moore, 1971) but the doses required exclude its use in human therapy. Rats have been shown to be insensitive to the drug (Strombeck, 1971). Cycloheximide is thought to exert its inhibitory effects by interfering with the enhanced sodium flux from plasma to lumen due to cholera toxin. (Grayer, Serebro, Iber & Hendrix, 1970; Harper, Grayer, Yardley & Hendrix, 1970).

Intraluminal administration of acetazolamide also reduces the effect of toxin challenge (Norris, Curran & Schultz, 1969). Salt solutions containing glucose perfused intraluminally are also beneficial and when used in combination with acetazolamide, are capable of abolishing fluid loss. These procedures may be of limited application clinically as this treatment would necessitate hospitalization and careful supervision of the patient during therapy.

Erythrose inhibits toxin production by *V. cholerae* (Chowdhury & Datta, 1965). Its effect on toxin-induced diarrhoea has not

been assessed. The use of this chemical in the prophylactic treatment of cholera has not been investigated. It has been shown to prevent diarrhoea in adult and infant rabbit models challenged with viable *V. cholerae* organisms (Bhattacharja, Chowdhury and Datta, 1965).

Loxomboom and his associates were able to inhibit the inflammatory response induced by cholera toxin in the mouse foot oedema model using protein synthesis inhibitors and anti-inflammatory drugs. Aspirin was ineffectual and indomethacin was capable of reducing the response to cholera toxin by 50%. (Loxomboom, Goth & Finkelstein, 1971). Finkelstein was able to inhibit and reverse oedema in the rat foot following toxin challenge using cycloheximide (Finkelstein, Jehl and Goth, 1969).

Escherichia coli toxin-induced diarrhoea

The participation of *E. coli* in pathological processes in the intestine has only been appreciated in the last twenty years. Some strains of this bacterium can be isolated from the intestinal tract of healthy individuals but there is a marked paucity of their numbers in the upper small intestine. In disease states the organisms populate the entire intestinal tract and the upper bowel is particularly striking in this respect. This alteration in distribution of the bacteria was first reported by Thomson in 1955. It has been suggested by Banwell and Sherr that the organisms may find their way into the small intestine by retrograde spread from the lower regions of the gut as well as by exogenous infection per os (Banwell & Sherr 1973). Pathogenic strains of *E. coli* have been implicated in human

adult and neonatal diarrhoeas (Neter, 1959; Ewing, Davis & Montague, 1963) in swine, piglet and neonatal calf diarrhoeas (Nielson & Sauter, 1968; Smith, 1962; Smith & Halls, 1967a, b). Sojka concluded that most cases of neonatal diarrhoea in piglets were attributable to coliform infection (Sojka, 1965, 1971) but in the calf, bacteria are not the main cause of diarrhoea. Smith was able to implicate *E. coli* in 7 of 127 unrelated cases of neonatal diarrhoea that he investigated (Smith, cited in Bywater, 1974).

There is a sharp distinction between *V. cholerae* and *E. coli* organisms with respect to their ability to initiate diarrhoea. In the case of coliform organisms there are at least two separate processes which can lead to the loss of fluid and electrolytes from the intestine. Both invasive and non-invasive strains of *E. coli* exist. The former induce secretory activity and damage the intestinal epithelium at the same time. The latter, however, are able to activate secretory mechanisms without any concomitant disruption of mucosal cell integrity (Sheahan, 1972). Some strains produce Salmonella-like and others Shigella-like diarrhoeas (Sakazaki, Tamura & Saito, 1967). Invasive organisms elicit inflammatory changes in the intestinal mucosa (Taylor et al, 1958; Yahagaki, Goda & Sasaki, 1967). The effects are seen within seven hours of infection (Ogawa, Nakamura & Sakazaki, 1968). Non-invasive strains do not have this capacity. De and his associates and Taylor's group were unable to demonstrate inflammatory reactions with non-invasive strains (De et al, 1956; Taylor, Wilkins, Payne, 1961). These organisms are believed to produce their untoward effects via toxins (Gorbach, 1970). Thus the non-invasive, toxin-producing strains resemble *V. cholerae* in the manner in which they stimulate fluid and electrolyte losses in

the intestinal tract superficially at least. Only one mechanism is thought to be operative in choleraic diarrhoea, (see previous section).

The Toxins of Escherichia Coli

There are several reports of cell-free material prepared from cultures of *E. coli* being capable of exerting enterotoxic activity in the small intestine in animals. Bywater observed fluid and electrolyte loss in calf small bowel following challenge with a heat-stable cell free fraction (Bywater, 1970, 1973a, b). Pierce and Wallace stimulated secretory activity in dog and rabbit intestinal loops by introducing similar crude toxin fractions into the ligated loops (Pierce & Wallace, 1972). The preparation was thought to contain both heat-labile and heat-stable forms of the enterotoxin as the crude material was only partially heat-stable.

As do other gram negative bacteria, *E. coli* produce endotoxins but the importance of this toxin in producing diarrhoea is somewhat obscure. This toxin cannot evoke secretory responses if injected into intestinal loops in pigs (Smith & Halls, 1967a), nor in calves (Bywater, 1970). Endotoxin can however elicit hypersensitivity reactions in the intestine (Thomlinson, cited in Bywater, 1974) and stimulate inflammatory responses in pig gut (Kewworthy, 1974). Jacks and his associates were unable to separate enterotoxic and endotoxic activities by various separative techniques including gel chromatography and ultrafiltration when they used a crude toxin preparation as the starting material (Jacks, Wu, Braemer, Bidlach, 1973). Endotoxin is a lipopolysaccharide heat-sensitive component of the bacterial cell. Two enterotoxic moieties have been identified: a heat-stable and a heat-labile toxin (Gyles

& Barnum, 1969: Smith & Gyles, 1970a). The heat-stable toxin is associated with the extracellular medium when *E. coli* are grown in culture whereas the labile form is only detectable in cell lysate preparations. The latter toxin is a high molecular weight compound (M Wt. 5×10^6) but the former is a much smaller entity, its molecular weight being 1000-10000 (Banwell & Sherr, 1973: Bywater, 1970). The toxins also exhibit different antigenic properties. Heat-labile material can be neutralized by *E. coli* specific antisera in contrast to the heat-insensitive forms which cannot. This was demonstrated by Rutter and Luther in 1973. Rabbit loop sensitivity to the toxins is a further means of differentiating between the two types of enterotoxin. The model is susceptible to challenge by the heat-sensitive toxin but not to the heat-stable form (Smith & Gyles, 1970b). The onset of diarrhoea occurs earlier with the heat-stable toxin and the effects are already apparent two hours after infection. The heat-labile toxin does not produce any measurable effects for a further one or two hours, (Larrivière et al, 1972). It has been suggested that the two forms of enterotoxin may, in fact, be a single enterotoxic substance. There are several objections to this hypothesis of Smith and Gyles (Smith & Gyles, 1970b). Although all strains of *E. coli* investigated and found to be enterotoxic in pigs are capable of synthesising the heat-stable toxin, not all strains produce heat-labile forms. The possession of the K 88 antigen appears to be associated with this property of *E. coli* strains. Gyles and Barnum noted that more enteropathogenic strains produced heat-labile toxin alone. Heat-labile toxin from one strain which was enterotoxic in pigs was a more

potent stimulant of fluid secretion in the rabbit loop model than the heat-stable toxin produced by the same strain (Smith & Gyles, 1970b). Heat-labile toxin is a much larger molecule than the heat-stable toxin and causes death by endotoxic shock rather than by dehydration which is the lethal factor in heat-stable toxin syndromes. The work of Kohler has provided these results (Kohler, 1971). It is feasible therefore that the larger heat-labile toxin contains the endotoxic moiety in its structure. In support of the single enterotoxin theory is the observation that both toxins are transferred on the same ent-plasmid during bacterial replication and both are resistant to the effects of trypsin, and neither is adsorbed on to kaolin nor aluminium hydroxide (Sheahan, 1972). Skin permeability enhancing activity is not associated with either toxin, although one strain of *E. coli* (08: K87, K88a, B:H19) has been found to possess such activity (Moon & Whip, 1971: Glew, Gorbach, Sack & Wallace, 1969: Sack, Gorbach, Banwell, Jacobs & Chatterjee, 1971).

Toxins have been produced by various methods (Smith & Halls, 1967b: Kohler, 1968: and Gyles & Barnum, 1969).

Experimental Models

(a) Rabbit Ligated Loop Model.

This model has been employed to assess the effects of cell-free preparations of *E. coli* cultures on gastro-intestinal function. Zinnaka and Carpenter investigated the nature of the fluid losses and the electrolyte composition and protein concentration in the secreted material (Zinnaka & Carpenter, 1972). De, Battacharya and Sarker tested a wide range of *E. coli* strains for enteropathogenicity

in this model and found good correlation between the ability of the strains to cause diarrhoea and to stimulate fluid production in the intestinal loops (De et al, 1956). All three strains of coli isolated in infantile diarrhoea and fifteen of the twenty strains obtained from adult intestine in patients with acute diarrhoea were found to give positive loop lesions. Taylor and Maltby together with Payne observed that the proximal small intestine was more sensitive to the toxin than more distal segments. (Taylor et al 1958). McNaught and Roberts (1958) showed that positive loop lesions could be elicited with coliform bacteria isolated from human patients but Taylor, Wilkins and Payne were not able to confirm the usefulness of the model when they tested the effects of *E. coli* strains isolated from other animals and which had been shown to be enteropathogenic in those species (Taylor, Wilkins and Payne, 1961). Other interesting observations on the locus of isolation of the organisms and their ability to stimulate fluid production in this model were made by Namioka and his colleagues (Namioka et al, 1958). Namioka demonstrated that whereas fourteen of the fifteen strains isolated from the ileum of pigs dying of transmissible viral enteritis were able to cause fluid secretion, none of the ten obtained from the colon were active. Larrivière has used the model to distinguish the two different types of enterotoxin (Larrivière et al, 1972). Rabbit jejunal loops have been used by Glew and by Sherr's groups to investigate various aspects of *E. coli*-induced diarrhoea (Glew, Gorbach, Sack & Wallace, 1969; Sherr, Banwell & Hendrix, 1971).

(b) Pig Intestinal Models.

Both ligated and perfused loop systems have been developed to investigate the changes that occur in intestinal handling of water and electrolytes (Bywater unpublished observations). The ligated loop has also been used by Moon and his associates (Moon et al, 1971).

(c) Thiry-Vella Loop Models.

These loops have been employed in dogs (Pierce and Wallace, 1972) and in neonatal calves (Bywater, 1970, 1973a) to follow the changes in fluid and electrolyte behaviour after the administration of *E. coli* toxin preparations.

(d) Ligated Intestinal Loops in Calves.

This type of experimental model has been used by Bywater to follow the fluxes of water and inorganic ions at various levels in the intestine in healthy and infected animals. (Bywater & Jeffries in preparation, Bywater, 1973b).

Mechanism of Action of *E. coli* Toxin

Escherichia coli toxin resembles cholera toxin in that it fails to produce any overt histopathological changes in the intestinal mucosa either in animal or in human patients (Moon et al., unpublished observations cited in Sheahan, 1972: Kohler & Cross, 1969: Banwell, Gorbach, Pierce & Mitra, 1971). It has been suggested that the K88 antigen secures the attachment of the bacteria to the epithelial surface of the intestine by penetrating the surface layer of mucus (Jones & Rutter, 1972). Colonization of the upper small intestine and toxin production are essential to the pathogenic mechanism (Sheahan, 1972).

The nature of the fluid secreted in *E. coli* diarrhoea is identical to that produced in response to cholera toxin challenge both in ionic composition and in the relative absence of proteinaceous material. It also shows the same isotonicity to plasma as the choleraic fluid. These results were obtained in dogs (Pierce & Wallace, 1972) and in rabbit and pig intestinal loop models (Moon, Whipp, Engstrom & Baetz, 1970).

The similarity of the anatomical lesions or rather the lack of them and the biochemical changes which occur in these bacterial diarrhoeas is highly suggestive that the same mechanism is responsible for the symptoms. Like cholera toxin, *E. coli* toxin has been shown to stimulate adenyl cyclase activity in the intestinal tissue (Guerrant, Chen & Sharp, 1972; Kantor, Kao & Gorbach, 1974). Further support for the hypothesis of a common mechanism is afforded by the work of Sherr. He and his colleagues were able to inhibit *E. coli*-induced fluid loss by cycloheximide pretreatment (Sherr, Banwell & Hendrix, 1971). Not all the evidence appears to give support to this theory however. Glucose absorption in some species is unaffected by exposure of the intestine to toxin, namely, in the calf (Bywater, 1970) and in the dog (Pierce & Wallace, 1972) but in the rabbit gut as in the piglet glucose absorption seems to be depressed by *E. coli* enterotoxin (Sherr, Banwell, Rothfield & Hendrix, 1973; Bywater & Jeffries in preparation). It is possible that these results reflect species differences in the response to toxin challenge rather than differences in the mode of action of the toxins per se. The response of pig and rabbit intestine to cholera toxin with regard to the effects on glucose absorption under the same experimental conditions as the coliform challenge has not been reported. Other evidence which casts doubt

on this hypothesis is the difference in the time course of the cholera and *E. coli* toxin effects. The response to *E. coli* toxin is of much shorter latency and is more transient than that to cholera toxin challenge. The fact that pretreatment of tissues with cholera toxin reduces the response to *E. coli* toxin is in favour of their having a common mechanism of action (Pierce & Wallace, 1972).

Neither toxin has an immediate effect on adenylyl cyclase activity although the latent period is shorter in the case of the *E. coli* toxin. It is conceivable, therefore, that both these toxins may stimulate the release or synthesis and subsequent release of a mediator or local hormone that is more directly responsible for the fluid and electrolyte losses. The putative mediator would, in addition to being locally available, have to be capable of mimicking the effects of toxin challenge and in general would have to fulfil criteria similar to those which have to be met with by postulated transmitter substances at neural endings (e.g. for acetylcholine).

Part 3. Prostaglandins and Bacterial Diarrhoeas

Nomenclature

C_{20} polyunsaturated fatty acids are the precursors of a relatively new group of lipid hormones, the prostaglandins. The parent compound is prostanoic acid, the structure of which is shown in Figure 1.

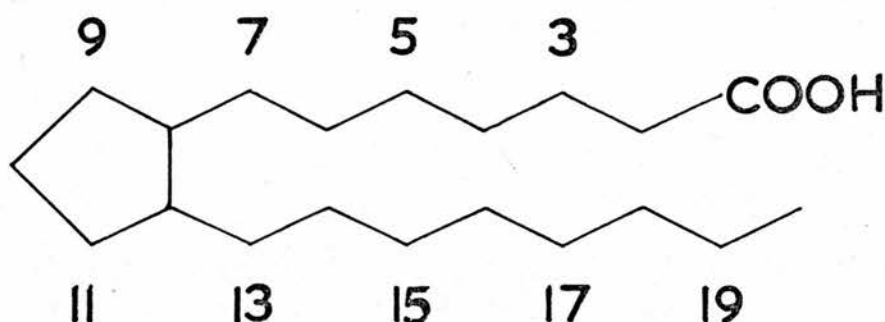


FIGURE 1. PROSTANOIC ACID

All prostaglandins are related to this acid and may be named systematically on the basis of their relationship to this structure (Nugteren, van Dorp, Bergstrom & Hamberg, 1966), although they are more usually referred to by their trivial names. There are eight series of prostaglandins that are known to occur in mammalian tissues: the smooth muscle stimulant substance of Goldblatt (Goldblatt 1933) and von Euler (1934) containing prostaglandins of the F and E series (PGF and PGE); PGA and PGB, the products of alkali treatment of PGE compounds; the prostaglandin

intermediate PGC which is formed from PGA by the action of prostaglandin A isomerase (Jones, 1972); PGD, also the product of an isomerase-mediated reaction but derived from PGR series of prostaglandins (Foss et al, 1973); the two PGR groups of compounds, 15 hydroperoxy PGR (also termed PGG) and PGR (also named PGH - See Samuelsson, 1973) which were identified by Nugteren and Samuelsson independently (Nugteren & Hazelhof, 1973; Samuelsson, 1973).

With the exception of the R series, all prostaglandins have two structural features in common namely, the C_{13-14} double bond and the hydroxy group at C_{15} . All of the prostaglandins exhibit differences in the cyclopentane ring. These are illustrated in Figure 2.

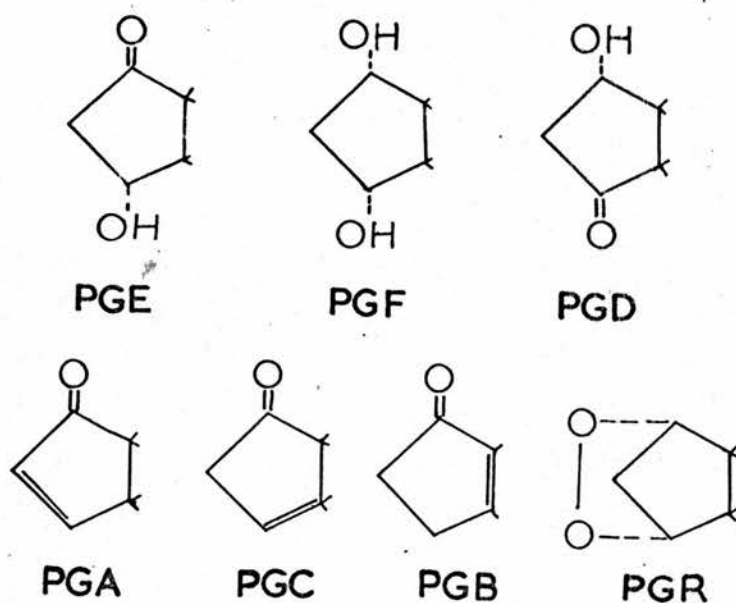


FIGURE 2. Structural differences in the prostaglandin ring.

There are two side chain units composed of C_{1-7} and C_{13-20} carbon atoms and these are referred to as the α and the ω chains

respectively. In the B prostaglandins the α chain is in the transfiguration whereas it occurs in the cis position in all the other series. In the PGF groups epimerization at the C₉ hydroxyl position is found. This gives rise to the α and β types of PGF. Only the F α prostaglandins are known to occur naturally and are thought to be biologically significant.

There are several centres of asymmetry in the prostaglandin molecule. Thus a large number of isomers may be synthesised chemically (Caton, 1972). Only one unusual isomer is known to occur naturally, the 15-epi-PGA₂ isomer (Weinheimer & Spraggins, 1969).

There are 3 sub-divisions for each group of prostaglandins. These are named according to the number of double bonds in the side chain part of the molecule. In the 1 series only one such bond is present in the structure - the C₁₃₋₁₄ double bond. In the 2 sub-group another is found at the C₅₋₆ position, and in the 3 series the third is contained in the chain at C₁₇₋₁₈.

19 hydroxy prostaglandins of the A and B type are found in human semen but whether these should be regarded as separate groups or as metabolites of PGA and PGB is not known. (Bygdeman & Samuelsson 1964, 1966).

The structure of some of the naturally occurring prostaglandins is shown in Figure 3.

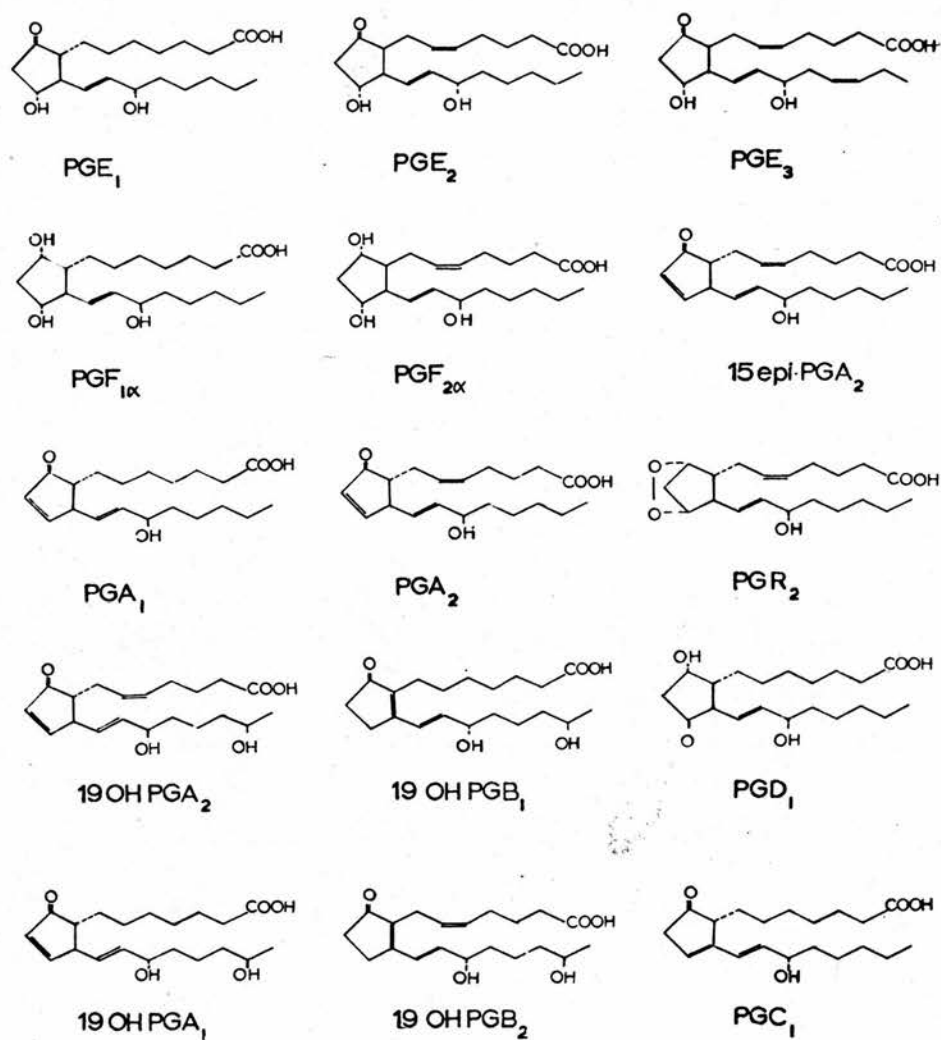


FIGURE 3. Some naturally occurring prostaglandins.

Synthesis

Prostaglandin storage is not a widespread phenomenon. The level of hormone found in tissues is much lower than that which can be released by an appropriate stimulus. Thus a readily available enzyme system together with an adequate supply of precursors is of prime importance for prostaglandin synthesis.

The enzyme responsible for this synthesis is associated with the microsomal fraction of cells and is present in a large number of tissues from various organs (van Dorp, 1966). The richest source is seminal vesicle tissue from sheep (Nugteren, Beerthius, van Dorp, 1966 & 1967; Bergstrom, Danielsson & Samuelsson, 1964) and ox (Wallach, 1965). Studies with these preparations indicate that the type of prostaglandin formed and the relative amount of each may be influenced by several factors: The presence or lack of co-factors (Samuelsson, Granstrom & Hamberg, 1967; Yashimoto, Ito & Tomita, 1970); the addition of hormones, e.g. oestrogens and progesterone (Poyser, 1974), catecholamines and biogenic amines (Foss, Sih, Takeguchi & Schnoes, 1973). Giroud has shown that in inflammation the ratio of PGE to PGF increases (Giroud et al, 1974). There is circumstantial evidence that PGR may be the unstable active principle of rabbit aorta contracting substance (Gryglewski & Vane, 1972; Samuelsson & Hamberg, 1973) released from traumatized guinea-pig lung (Piper & Vane, 1971). The mechanism whereby polyunsaturated fatty acids are converted into prostaglandins has been elucidated (see Review by von Euler, 1967; Horton, 1972 and Caton, 1972). Tissues from different sources show preference in the type of prostaglandin they synthesize normally.

Distribution

Prostaglandins are widely distributed in animal tissues and in fluids including intestine, lung, heart, smooth muscle, endometrium, skin, brain, iris, semen and menstrual fluid (see Reviews and Monographs - Bergstrom, Carlson & Weeks, 1968; Horton 1969, 1972, Karim 1972 and Cuthbert 1973). The Gorgonian coral is the only non-mammalian, non-amphibian source of prostaglandin - it has been found to contain significant quantities of 15-epi PGA_2 and its methyl ester. (Weinheimer & Spraggins, 1969; Bundy, Lincoln, Nelson, Pike & Schneider, 1971).

Release

Spontaneous and evoked release of prostaglandins has been observed in a wide variety of tissues in many species (see Table 1). A physiological milieu is not a prerequisite for prostaglandin release, and release of these compounds is often found in pathological conditions. Thus in some inflammatory conditions e.g. uveitis and conditions resembling uveitis produced by mechanical stimulation in rabbits, and uveitis in man (Ambache, Kavanagh and Whiting, 1965; Eakins, Whitelocke, Perkins, Bennett and Unger, 1972; Eakins, 1973), allergic dermatitis and inflammatory skin conditions (Greaves, McDonald-Gibson and S ndergaard, 1971) prostaglandins are released in greater amounts into perfusates as compared to the amounts found in the perfusate of normal uninflamed tissues. Burn injuries in man and experimental animals are associated with enhanced output of PGE predominantly into blister fluid and lymph draining from the affected site (Jonsson and Hamberg, 1973). The PGE-like material was conclusively identified as PGE_2 by gas chromatography-mass

Table 2. Release of Prostaglandins

Species	Tissue	Stimulus	Reference & Stimulus
Rat	Stomach (Mucosa)	1) Vagal stimulation, 2) Spontaneous, pentagastrin theophylline 3) Histamine	Bennett et al, 1967, 1 Ramwell & Shaw, 1968a, 1 Ramwell & Shaw, 1968b, 1, 2, 3 Shaw & Ramwell, 1968a, 1
	Stomach Serosa	1) Spontaneous, vagal transmural stimulation 2) Carbachol, 5 hydroxy- tryptamine (5HT) 3) nerve stimulation, stretch	Bennett et al, 1967, 1 Coceani, Pace-Asciak, Volta & Wolfe, 1967, 1 Coceani, Pace-Asciak, Wolfe, 1968, 1, 2
	Skin	Inflammation Carag��enin	Willis, 1969
	Lung	Air Embolus	Alabaster & Bakhle, 1970
	Intestine	Spontaneous	M.J. Dlugolecka (unpublished observations)
Frog	Intestine <u>in vitro</u>	1) Distilled water 2) Spontaneous	Vogt & Distelkottter, 1967, Bartels, Vogt & Wille, 1968, 2
	Intestine <u>in vivo</u>	Acetyl choline, phospho- lipase A, spontaneous, dimethyl- phenylpiperazonium iodide	Bartels, Kunze, Vogt & Wille, 1970
Guinea- Pig	Lung (a) whole, perfused	1) Anaphylaxis 2) particles 3) Phospholipase A	Piper & Vane, 1969, a, b, 1 Lindsey & Wyllie, 1970, 2 Palmer, Piper & Vane, 1970b, 2

Table 2 - contd.

Species	Tissue	Stimulus	Reference & Stimulus
Guinea-Pig	Lung (a) whole, perfused	4) Histamine 5) Tryptamine, 5HT 6) Massage 7) Distension 8) Air embolus	Piper & Vane, 1971, 2, 8 Vogt et al, 1969, 3 Alabaster & Bakhle, 1970 & Unpub., 4, 5 Berry, Edmonds, Wyllie, 1971, 7
	Lung (b) chopped	Stirring	Palmer, Piper, Vane, 1970a.
	Uterus	Distension	Poyser, 1971
Dog	Spleen	Particulate material	Gilmore, Vane, Wyllie, 1969
	Bladder	Distension	Gilmore, 1968
	Skin	Burn injury	Jonsson, 1970
Rabbit	Eye (anterior chamber)	1) Mechanical 2) Inflammation	Ambache et al, 1965 Eakins, Whitelock, Perkins, Bennett and Unger, 1972
	Epigastric fat pad	Hormonal	Lewis & Matthews, 1969
	Intestine	Spontaneous, diarrhoea	Thesis Section 1
	Leukocytes	Phagocytosis	Higgs & Youlden, 1972
Human	Thyroid	Carcinoma	Sandler et al, 1969, Williams et al, 1968.
	Uterus	Partuition	Karim, 1968

Table 2 - contd.

Species	Tissue	Stimulus	Reference & Stimulus
Human	Eye	1) Uveitis 2) Acute angular glaucoma	Eakins et al, 1972 Wyllie & Wyllie, 1971
	Lung	1) Anaphylaxis 2) Agitation	Piper, 1972
	Skin	1) Inflammation 2) Burn	Greaves et al, 1971 Jonsson & Hamberg, 1973

spectrometry by Arturson, Hamberg and Jonsson (1973). Tumours of the thyroid and certain other tumours of the fore and mid-gut are thought to release prostaglandins into the blood stream and it is possible that some of the side effects experienced by patients may be attributable to this (Sandler, Karim and Williams, 1968; Sandler, Williams and Karim, 1969). Conclusive identification of the prostaglandin, which resembles PGF_2 in its thin layer chromatographic and biological activity, is however lacking. Prostaglandin release from rat stomach has been demonstrated by Bennett and his colleagues and by Pace-Asciak, Morawska, Coceani and Wolfe (Bennett, Friedmann and Vane, 1967; Pace-Asciak, Morawska, Coceani and Wolfe, 1968a). Mucosal release exceeds that of the serosal and muscle tissues (Wolfe, Coceani, Pace-Asciak, 1967).

Prostaglandins and the Gastro-intestinal tract

Prostaglandins are found throughout the entire length of the gastro-intestinal tract in man, pigs, rats, guinea-pigs and rabbits (Table 3). Little is known about the relative distribution in the individual layers of the gut wall, although Bennett and his associates found that in human stomach, the highest concentrations are contained in the mucosal layer (Bennett, Murray and Wyllie, 1968a). It is likely that strain and species differences occur in the type and relative amounts of prostaglandins present in the gut. Coceani and Pace-Asciak together with Wolfe detected PGE_1 , PGE_2 , $\text{PGF}_{1\alpha}$ and $\text{PGF}_{2\alpha}$ in perfusates following response to nerve stimulation of rat stomach (Coceani, Pace-Asciak and Wolfe, 1968). Bennett found PGE_1 and some PGF_1 only in a series of experiments in which the release of

prostaglandins into luminal perfusates of rat stomachs during nerve stimulation was investigated (Bennett, Friedmann and Vane, 1967). The characterization of the prostaglandin was by thin-layer chromatography and biological assay in both instances. Shaw and Ramwell observed release of PGE_1 , $\text{PGF}_{1\alpha}$ and $\text{PGF}_{2\alpha}$ from stimulated rat stomach (Shaw and Ramwell, 1967).

Prostaglandin compounds affect motility and secretory activity in the gastro-intestinal tract.

Site	Species	Reference
Stomach	Man	Bennett, Murray & Wyllie, 1968a
	Rat	Bennett et al, 1967 Coceani, Pace-Asciak, Volta, Wolfe, 1967
Intestine	Frog	Vogt, Suzuki & Babilli, 1966
Small intestine	Man, rat	Bennett & Fleshler, 1970 unpublished observations
	Rat, calf cat, pig, rabbit	M. Dlugolecka, unpublished observations. Thesis Sections 1 & 2
	Guinea pig, rabbit	Ambache, Brummer, Rose & Whiting, 1966
Large intestine	Man, rat, guinea pig	Bennett & Fleshler, 1970
	Pig, calf	M. Dlugolecka, unpublished observations
	Pig	Miyazaki, 1968

Table 3. The occurrence of prostaglandins in the gastrointestinal tract.

Prostaglandins and Gastro-Intestinal Motility

In vitro PGE and PGF contract whole segments of intestine in most species investigated, although in guinea-pig colon and rat jejunum relaxation may precede contraction (Bennett & Fleshler, 1969; Bergstrom, Duner, von Euler, Pernow & Sjoval, 1959). Circular muscle responds to PGE_1 and PGE_2 by relaxing or by inhibiting the effect of other stimulants, e.g. acetylcholine (Bennett, Eley & Scholes, 1968b; Bennett & Fleshler, 1970). PGF causes contraction of both longitudinal and circular muscle in guinea-pigs, rat, and man (Bennett & Fleshler, 1970), and in dogs (Vanasin, Greenough, Schuster, 1970). PGE contracts longitudinal muscle in human stomach (Bennett et al, 1968a). The contractions produced by PGE and PGF compounds differ qualitatively; that of the F series being slower in onset and taking longer to reach the peak height at a given dose (Horton & Main, 1965). Opposite effects are observed with these two prostaglandins on peristaltic activity induced by increasing intraluminal pressure. PGE_1 inhibits peristalsis when applied serosally whereas PGF enhances peristaltic contraction of circular muscle (Bennett & Fleshler, 1970).

In vivo intravenous injection of a mixture of prostaglandins causes an increase in intestinal motility in rabbits and mice (von Euler, 1966). PGE_1 and PGE_2 given by either intravenous or intra-arterial injection to rats contract intestinal muscle and increase intraluminal pressure (Bennett, Eley & Scholes, 1968c) but in dogs inhibition of vagally induced contraction occurs (Chawla & Eisenberg, 1969). A similar inhibitory effect is seen in rats (Main, unpublished observations, Main 1972). Intra-arterial injection of $\text{PGF}_{2\alpha}$ in dogs, via the Superior Mesenteric artery,

produces contraction of the jejunum.

In man, intravenous infusion of PGE results in abdominal cramps in the subjects (Bergstrom et al, 1959; Carlsson, Ekelund & Oro, 1968). Horton et al noted increased intestinal motility in human volunteers given doses of PGE₁ totalling 0.8-3.2 mg orally. Sensations of mild colicky pain and enhanced motility were experienced by the participants (Horton, Main, Thompson & Wright, 1968). Misiewicz carried out objective investigations and observed that there was a delay of 30-120 min. in onset of effects and that their duration was of the order of 1-6 hours. There was marked increase in contractile activity of both the small and large intestine. The decrease in transit rate could have been attributable in part to the increase in volume of intestinal content - secretion of watery fluid occurred contemporaneously. (Misiewicz, Waller, Kiley & Horton, 1969). Diarrhoea in carcinoma patients can, in some cases, be correlated with the enhanced levels of prostaglandin in peripheral venous blood (Williams, Karim and Sandler, 1968).

Prostaglandin and Gastro-Intestinal Secretions

The effect of prostaglandins on secretory activity in the gastro-intestinal tract depends on the region that is being investigated. In the stomach PGE₁ inhibits the secretion of gastric acid, an inhibition of adenylyl cyclase activity being implicated in the response (Robert, 1968; Shaw & Ramwell, 1967). There are reports both favouring and disputing the involvement of 3'5' cyclic adenosine mono phosphate (Wilson, 1972). An inhibitory effect of prostaglandins on the adenylyl cyclase system

is also found in toad bladder, renal tubule and lipocytes (Orloff, Handler, Bergstrom, 1965; Orloff & Grantham 1967; Steinberg & Vaughan, 1967 and Hittelman & Butcher, 1972).

In the intestine Pierce and his collaborators found that PGE_1 reduced the absorption of fluid and electrolytes in a dose dependent manner when applied to the luminal aspect of Thiry-Vella loops. Doses of $2\mu\text{g}$ - $24\mu\text{g}/\text{minute}$ or $1\mu\text{g}$ - $12\mu\text{g}/\text{ml}$ were used. Injection via the Superior Mesenteric artery had similar results but the dose required to elicit a maximal secretory response was very much lower - $24\mu\text{g}/\text{min}$. PGA_1 and $\text{PGF}_{2\alpha}$ had qualitatively similar effects as PGE_1 although $\text{PGF}_{2\alpha}$ was more potent and enhanced the protein content of the intestinal secretions. The response to cholera toxin was studied in these animals and compared with that to prostaglandin administration (Pierce, Carpenter, Elliott & Greenough, 1971). A direct mucosal interaction was likely because isolated ileal mucosa can be induced to secrete chloride ions and diminish the absorption of sodium ions by adding either PGE_1 or PGA_2 to the bathing fluid (Al Awqati, Field, Pierce & Greenough, 1970b; Greenough, Pierce Al Awqati & Carpenter, 1969). PGE_1 has similar effects on ion secretion in isolated mucosa from rabbits and man to theophylline, dibutyryl CAMP, and cholera toxin (Al Awqati, Cameron, Field, Greenough, 1970a). Prostaglandins stimulate adenylyl cyclase activity in the gut (Kimberg, Field, Johnson, Henderson, Gershon, 1971; Sharp & Hynie, 1971). Matuchansky and Bernier demonstrated that perfusion of isolated segments of intestine with PGE_1 inhibited the absorption of water and electrolytes (Matuchansky & Bernier, 1971).

The ability of prostaglandin compounds to mimic the effects of cholera toxin in the gut and the observation that intestinal

adenyl cyclase activity is enhanced by *E. coli* toxin (Raskova, 1974), cholera toxin and prostaglandins might have been indicative of prostaglandin involvement in the diseases associated with *V. cholerae* and *E. coli* infection.

Anti-Inflammatory Drugs and Prostaglandins

Steroids and non-steroidal anti-inflammatory agents suppress inflammatory processes by independent mechanisms. That modified by steroid drugs remains a mystery but the mechanism of action of non-steroidal compounds is better understood. These latter agents are believed to ameliorate inflammatory states by interfering with prostaglandin biosynthesis. Prostaglandins are involved in the later stages of inflammation in carageenin-induced oedema in rats (Willis, 1969). PGE_1 activity increased after a latency of several hours, reaching peak levels at about 16 hours after administration of the irritant.

Prostaglandin synthetase activity is suppressed or inhibited by anti-inflammatory agents in dog spleen (Ferreira, Moncada & Vane, 1971; Flower, Gryglewski, Herbacinska-Cedro & Vane, 1972), guinea-pig lung (Vane, 1973) and uterus (Poyser, 1974), in rabbit brain (Flower & Vane, 1970) and kidney (Davis, 1971; Davis & Horton, 1972), in human skin (Greaves & McDonald & Gibson, 1972; Ziboh, McElligot & Hsia, 1972), and in platelets (Smith & Willis, 1971). Synthesis is abolished or at least markedly reduced in bovine seminal vesicles (Tomlinson, Ringold, Qureshi and Forchielli, 1972). Vane (1971) has suggested that the beneficial effects of Aspirin and Indomethacin ^{are} due to the suppression of prostaglandin synthesis.

The sensitivity of all the above tissues is not identical

with respect to the actions of the anti-inflammatory drugs but there appears to be a good correlation between the order of potency of the drugs as prostaglandin biosynthesis inhibitors and their clinical effectiveness (Flower, et al, 1972). In dog spleen a variety of drugs was studied and their ability to inhibit inflammation was found to reflect their anti-prostaglandin activity. The ID_{50} of anti-inflammatory drugs was observed to be lower than the plasma concentration likely to be achieved on a therapeutic dose regime in most instances, even allowing for plasma protein binding (Flower et al 1972).

Very high doses of prostaglandins are themselves capable of exerting anti-inflammatory actions. It has been suggested that steroid release may be involved in this situation (Glenn & Rohloff, 1972).

There is much circumstantial evidence to implicate prostaglandins in the initiation of fluid and electrolyte loss observed in clinical and experimental diarrhoea in man and in animals. This thesis was embarked upon with the intention of testing this hypothesis. The scheme envisaged for prostaglandin mediation of the bacterially induced diarrhoeas is outlined in Figure 4. Toxins prepared from *V. cholerae* strains and *E. coli* heat-stable toxin were employed to simulate the clinical condition in several experimental models and the answers to the following questions were sought:

- (1) Is prostaglandin release associated with the fluid and electrolyte secretion induced by the bacterial toxins of *V. cholerae* and *E. coli* or is it a reflection of non-specific trauma incurred by the intestinal epithelium?

(2) Do the toxins contain prostaglandins or do they enhance the synthesis of prostaglandins?

(3) Is the fluid secretion induced by the toxins blocked by inhibitors of prostaglandin synthesis and particularly Indomethacin?

(4) Is a reduction in prostaglandin synthesis related in a dose-dependent manner to the reduction in the diarrhoeic response to toxin challenge?

(5) Can prostaglandins mimic the effects of toxins on the pattern of ion and water movement in the small intestine? (This aspect was only investigated in the case of E. coli toxin).

If prostaglandins are ^{the} mediators of the enteropathogenic effects of the non-invasive enterotoxins then the changes in ion and water movement across the intestinal mucosa should be identical.

To prove that prostaglandins were causally related to the bacterial toxin induced diarrhoeas would afford new approaches to the clinical treatment of the syndrome which might be more effective than those tried hitherto. (Bennett (1971) has suggested that a trial of aspirin in the treatment of cholera would be worthwhile). To exclude their participation would also be of value in that it would serve to redirect therapeutic research in to possibly more fruitful avenues.

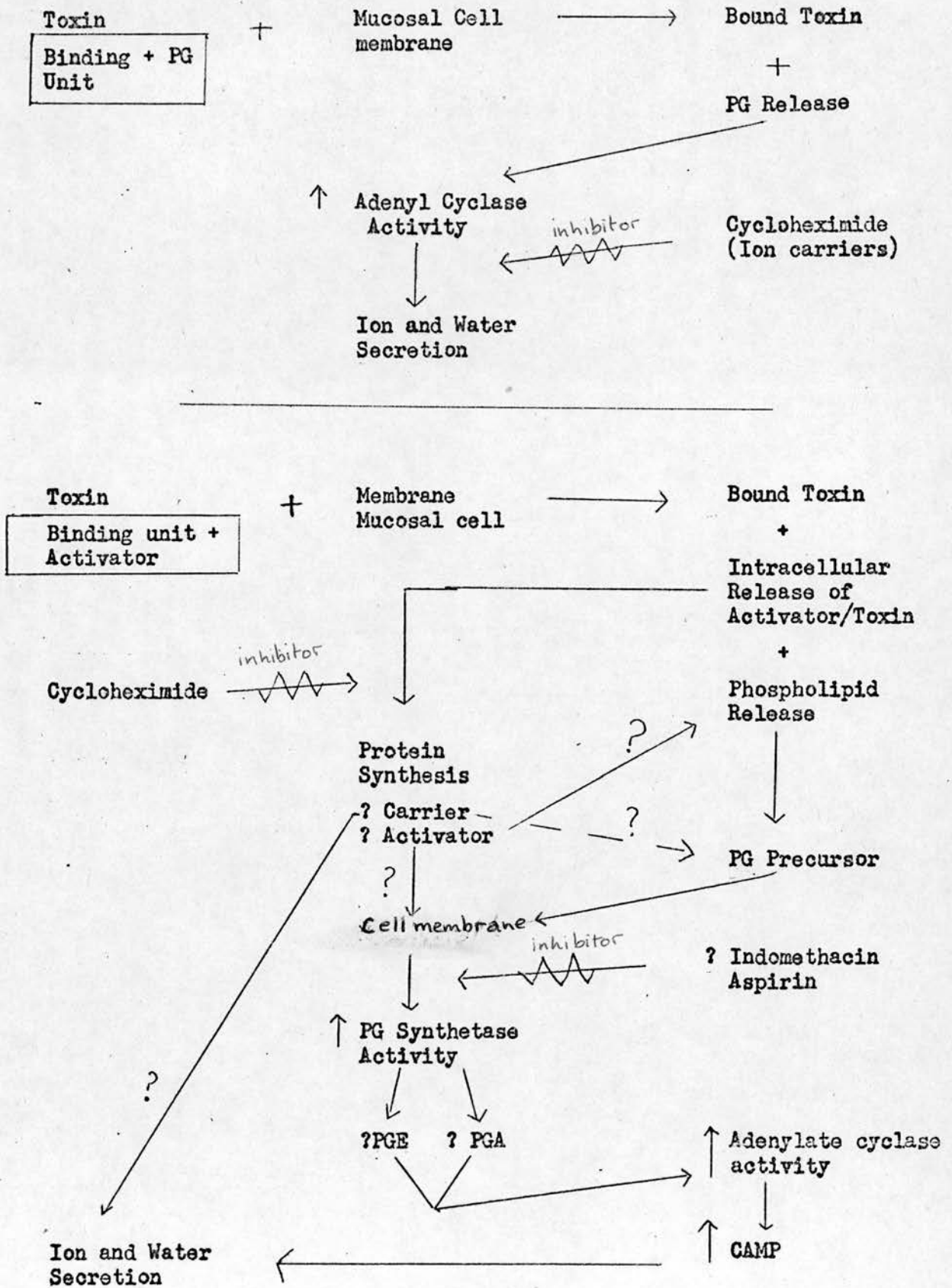


Figure 4.

Section 1. Part 1. The toxins used to simulate cholera in experimental animals.

The production of toxin by *V. cholerae* is thought to be essential to their enterotoxic effects although not all the strains isolated from human patients during outbreaks of cholera have been observed to elaborate toxins in in vitro culture. This probably reflects the specificity of their growth requirements rather than the absolute inability of that particular strain to produce enterotoxin (Craig, 1971; Sheahan, 1972). Some strain of vibrio, like *V. cholerae* 569B, appear able to synthesize toxins under a relatively wide range of growth conditions whereas others like *V. cholerae* strain 12 r (Ogawa) have much more stringent requirements (Latif, 1966; Sheahan, 1972).

The toxins used to investigate the role of prostaglandins in toxin-induced diarrhoea and in particular choleraic diarrhoea in experimental animals included two which were ready prepared, solid toxins. These were reconstituted as required. The third toxin preparation was produced by growing live vibrios in vitro. The solid toxins were crude Wyeth lot 002 toxin and highly purified freeze-dried toxin prepared from *V. cholerae* strain 225B. Wyeth toxin was donated by Dr. J. Seale, N.I.A.I.D., National Institute of Health, Bethesda, Maryland and was obtained by growing *V. cholerae* strain 569B on Richardson's TRY medium (Richardson, 1970). The lyophilized culture filtrate contained 2, 650,000 Bd (blueing doses) per gram solid material. Each 100g of the solid was prepared from approximately 8.45 litres of crude culture. The rabbit skin permeability test (Craig, 1965b) was used by the manufacturers (Wyeth Laboratories) to assess the potency of the material. The purified toxin was a gift from

Dr. Ungar, W.H.O., Switzerland and was produced by growing *V. cholerae* strain 225B on 3% peptone broth at pH 7.0 - 8.0. The culture filtrate was subjected to extensive purification using protein precipitation and chromatographic techniques. This material contained 1Bd per 12.5-15 μ g purified solid and the unit of rabbit loop toxicity was contained in 100 μ g of the purified toxin (Dr. Ungar - personal communication).

The third toxin used in these studies was that synthesised by *V. cholerae* 12r (Ogawa) grown on 5% Difco BactoPeptone medium containing 0.5% sodium chloride. This toxin was prepared during the course of the experimental work and, unlike the other two toxins, was stored as a liquid rather than as a solid. A method is described for the preparation of this toxin. The effects of incorporating antibacterial agents into the crude toxin-containing filtrate on the toxic activity are reported. An approximate estimate of toxin potency was obtained by comparing the volumes secreted by intestinal loops following challenge with the crude material and highly purified 225B toxin. The crude toxin solution contained 4.5 loop toxic units per millilitre - a 1.5 ml volume being equivalent to 600-800 μ g purified toxin. Slight variation between batches of crude toxin did occur.

The preparation of enterotoxin from *Vibrio cholerae* strain 12r (Ogawa) and the incorporation of antibacterial agents into the crude toxin-containing filtrate.

The method used to prepare the enterotoxin from *V. cholerae* strain 12r (Ogawa) was based on that described by Latif for the preparation of toxins using *V. cholerae* strains 569B and 12r (Ogawa).

Methods

(a) Toxin Production.

Vibrio cholerae strain 12r (Ogawa), isolated from a human patient in East Pakistan by Dr. J. Feeley and freeze-dried after serial passage through a suckling rabbit was redeemed from Latif's freeze-dried stock stored on Dorset egg slopes, and was used to seed a tube of Nutrient Broth Oxoid No. 2. The broth was incubated static at 37°C for 6 hours. This culture was then checked for growth of the organisms by phase contrast microscopy and subculture on Agar plates. The organisms were characterized as described by Latif in 1966. Subculturing also enabled the presence of other contaminating bacteria to be detected and to be eliminated by appropriate measures if necessary.

Difco-Bacto Peptone medium was prepared as a 5% solution containing 0.5% sodium chloride in volumes of 300 ml (Latif, 1966). The pH of the medium was adjusted to pH 7.4 with sodium hydroxide solution and any over-alkalinisation was corrected by the addition of hydrochloric acid.

A Winchester Quart bottle containing 200 ml of the above medium was seeded with a 5 ml aliquot of the Nutrient broth culture and was incubated at either 37 or 30°C for 18 hours. Constant movement of the culture was ensured by incubating in a Gallenkampf shaking water bath or by using a magnetic stirrer.

The incubates were then centrifuged at 4,000 rpm for 40 minutes at 4°C in an MSE major angle head centrifuge and the supernatant was filtered through Sartorius membranes at 4°C. The sterility of the filtrate was checked by incubating 1 ml aliquots in nutrient broth for 20 hours at 37°C. The remainder of the filtrate

was maintained at 4°C during this period.

In order to minimise the time interval between preparation and use of the toxin solution it was decided to incorporate anti-bacterial agents into the crude filtrate, provided that this procedure did not result in significant loss of enterotoxicity. The agents tested were Neomycin (100 µg/ml) and chlorhexidine as Hibitane solution (1:2000). This procedure rendered the final sterility check superfluous as both Neomycin and chlorhexidine were bactericidal in the concentrations used.

Control medium consisting of 5% Difco BactoPeptone medium containing 0.5% sodium chloride was autoclaved at 121°C for 20 minutes and allowed to cool. The sterility of the solution was checked by subculture techniques.

(b) Assay Systems

(1) The Rabbit Intestinal Loop Model

The rabbit ligated loop model has been used extensively to investigate the enterotoxic effects of crude cholera toxin preparations (Latif, 1966; Sheahan, 1972) and purified toxin (Ungar, personal communication). The technique has been described in detail (Latif, 1966; De and Chatterjee, 1953). A total of 8 rabbits was employed in this study: 5 were New Zealand White rabbits and 3 were Brown and Black Lops. The group included animals of both sexes and their weights ranged from 2.3 to 3.8 kg. All were deprived of food in the 24 hour period prior to toxin challenge. This procedure had the advantage of increasing the sensitivity of the animals to the effects of the toxin (Craig, 1971) as well as reducing the amount of food debris present in the small intestine. The rabbits were anaesthetised by the intravenous injection of sodium pentobarbitone, 40 mg per kg. At laparotomy the

small intestine was exteriorized and sections of terminal ileum 10 to 15 cm in length were tied off by means of Mersilk ligatures. A total of 8 to 12 loops was made in each animal, the last of these loops extending to a point some 15 cm proximal to the tip of the appendix. The rationale behind the use of this section of small bowel as opposed to other regions is discussed by Latif and Craig (Latif, 1966; Craig, 1971). Alternate loops were inoculated with 1.5 ml of toxin and control solutions but the maximum number of toxin-containing loops used in any one animal was 5 in order to ensure maximal secretory responses (Craig, 1971). The intestine was returned to the abdominal cavity taking care that the loops were not traumatized further. The incisions in the linea alba and the skin were sutured and the animals were allowed to recover from the anaesthetic. The rabbits were transferred to experimental cages for the remainder of the post-operative period. No food was given during this time. Animals were re-anaesthetized 9 to 24 hours after toxin challenge and the abdomen was incised once more. The small intestine was withdrawn very carefully and the loops were examined for signs of haemorrhagic necrosis. Any fluid present in the loops was aspirated and stored at -4°C or -15°C . The volume of the choleraic fluid was noted.

(b) Skin Induration Assay

The method is given in detail by Latif and described by Craig (Latif, 1966; Craig, 1965b). The fur on the rabbits' backs was shaved as close to the skin as possible. An area approximately 8 cm by 12 cm was marked out and subdivided into 4 sections. Intradermal injections of 0.1 ml and 0.2 ml toxin solution and control medium were made, each of the toxin and control doses being administered in diagonally opposite quadrants. The size of the



lesion at 8-12 hours after injection was noted. *The diameter of the lesion was measured in centimetres.*

Results

(a) The production of enterotoxin

Only filtrates obtained from peptone cultures incubated at 30°C were found to be enteropathogenic and to cause skin reactions on intradermal injection. None of the batches of toxin produced by incubating *V. cholerae* strain 12r at 37°C in peptone broth was able to stimulate fluid secretion in the ligated ileal loop in rabbits. Some of the material did, however, possess skin reactive substances - induration was detected following intracutaneous injection of 0.1 ml and 0.2 ml crude filtrate in 3 rabbits. A total of eight rabbits ~~was~~ injected with the toxin. Toxin prepared by incubating at 30°C for 18 hours (T30 toxin) was twice as potent a stimulant of induration as that prepared at 37°C (T37 toxin). An injection of 0.1 ml of T30 toxin produced a region of induration of 0.7 cm in diameter whereas injection of an equal volume of the T37 toxin resulted in a lesion of up to 0.5 cm in diameter. None of the three batches of T37 toxin was able to stimulate fluid production during the 9 to 24 hour experimental period. By contrast, the loops challenged with T30 toxin were found to be markedly distended with fluid by 10-12 hours and haemorrhagic necrosis of the intestinal wall was invariably present at 24 hours after toxin administration.

These results are summarized in Table 4.

(b) The effect of incorporating Neomycin (100 µg/ml) and chlorhexidine (1:2000 v/v) into crude cholera toxin filtrate.

Because T30 toxin alone was enterotoxic in ligated rabbit ileal loops, the effects of neomycin (100 µg/ml) and chlorhexidine (1:2000 v/v) were investigated using this toxin. A blind trial method was used

Table 4. The Skin Reactivity and Enterotoxicity of *V. cholera* 12r (Ogawa) Toxin Produced at 30°C and 37°C.

(1) Toxin Preparation	(2) Rabbit Strain and weight	(3) Skin Response	(4) Loop Response (to 1.5 ml)
T37 (a)	NZW 2.7 kg	-	- (inactive)
T37 (b)	NZW 3.5 kg	0.1 ml = +	- "
T37 (b)	NZW 3.1 kg	0.2 ml = ++	- "
T37 (b)	LOP 3.6 kg	-	- "
T37 (b)	LOP 2.5 kg	-	- "
T37 (c)	NZW 2.4 kg	T 37°C 0.1 ml = +	- "
T30°C		T 30°C 0.1 ml = ++	++ active
T37 (c)	LOP 2.6 kg	-	- (inactive)
T30°C	NZW 2.6 kg	0.1 ml = ++	++ active
T37 (c)		-	- (inactive)

- Key. 1. (a), (b), (c) refers to batches of toxin prepared separately by 37°C incubation of 5% Difco BactoPeptone cultures.
2. NZW refers to New Zealand White and LOP to Lop strains of animals respectively.
3. In skin reactivity estimations "+" indicates a lesion of 0.5 cm in diameter. "++" records a lesion of more than 0.7 cm maximum diameter. "-" indicates no lesion detectable.
4. "++" indicates fluid secretion of more than 5 ml fluid at 22 hours and the presence of haemorrhagic necrosis.

to assess the potency of toxin solutions containing these agents and comparing the activity with that of ordinary T30 toxin. No difference was detected in the ileal response to T30 toxin, T30 toxin containing neomycin and T30 toxin containing chlorhexidine. Neither chlorhexidine nor neomycin were able to stimulate fluid secretion independently when given by intraluminal injection to the ligated loop. The toxins were assessed in 2 rabbits.

Discussion

Only two previous reports of enterotoxin production using *V. cholerae* strain 12r (Ogawa) organisms have been encountered. Latif in 1966 used this strain to produce and study the skin permeability toxin in particular. In these investigations, significant amounts of enterotoxic activity were obtained by incubating the organisms in 5% Difco-BactoPeptone/0.5% saline medium at 37°C for 18 hours (Latif, 1966). Attempts to reproduce this method of toxin production were not successful. It is known that even slight variations in medium composition, pH and other parameters like surface to volume ratio and aeration, can greatly alter the amount of toxin produced (Evans & Richardson, 1968; Latif, 1966). Under the conditions of the experiment it was necessary to reduce the temperature to 30°C to obtain reasonable quantities of enterotoxic material. That total inhibition of toxin elaboration did not occur at higher temperatures can be seen from the ability of fresh T37 toxin to induce changes in skin permeability. Skin permeability factor, which is responsible for induration, is thought to be identical to enterotoxin possessing the same physical, chemical and antigenic properties as enterotoxin (Craig, 1971; Sheahan, 1972). The pH ^{of the culture medium} appears to be critical in determining the

release of enterotoxin into the medium. Evans and Richardson (1968) stated that a low initial pH was required for toxin production. Callahan and Richardson demonstrated that only when the pH of the medium rose to pH 7.4 did significant release of toxin material occur (Callahan & Richardson, 1973). The initial pH of the culture used was pH 7.4, therefore, release of toxin should not have been impeded by an acidic medium nor was it too alkaline to suppress toxin synthesis. It is possible that although significant toxin production occurred at 37°C, that the toxin rapidly deteriorated in the medium at this temperature. Cholera enterotoxin is unstable and activity falls off very quickly at such temperatures (Latif, 1966). A combination of high temperature (37°C) and initial alkaline medium (pH 8.0 or more) suppresses toxin production (Evans & Richardson, 1968).

The addition of neomycin 100µg/ml or chlorhexidine (1:2,000) does not diminish toxin potency. Neomycin, an antibacterial agent synthesized by *Streptomyces fradiae* is effective against both gram negative and positive organisms (Wilson & Schild, 1968) and thus would inhibit any residual *V. cholerae* growth and also kill any contaminating organisms in the intestinal loop itself. This drug has been used in *Escherichia coli* enterotoxin filtrates in concentrations of 100µg/ml. In this preparation, as in the case of cholera toxin, no significant decrease in enterotoxin activity was found (Smith & Halls, 1967b). Neomycin itself was unable to stimulate fluid secretion and, therefore, the fluid losses from the intestine in rabbits ^{were} ~~was~~ directly attributable to the effects of cholera enterotoxin. Neomycin is a protein synthesis inhibitor (Wilson & Schild, 1968). It is intriguing that other protein synthesis inhibitors like cycloheximide are

capable of interfering with the secretory processes stimulated by cholera toxin (Serebro et al, 1971; Moritz et al, 1971). Neomycin is poorly absorbed from the gastro-intestinal tract (Wilson & Schild, 1968). It is possible that its inability to modify choleraic secretions is due to the failure of the drug to reach intracellular sites in the intestinal mucosa. Like neomycin, chlorhexidine is also bactericidal. This compound is an organic halogen which in low concentrations is non-irritant to mucosal epithelium (Wilson & Schild, 1968). Thus it would not be likely to interfere with the secretory response of the intestine to cholera toxin by causing epithelial cell damage.

Crude cholera toxin and stool filtrates from human cholera patients are capable of causing induration and increasing skin capillary permeability in guinea-pigs and rabbits (Craig, 1965b). Induration and enhanced permeability are probably two manifestations of the same underlying vascular defect. Induration is probably as satisfactory a method of estimating toxin potency as the blueing effect seen after intravenous injection of Evans Blue dye at the site of toxin injection. Both techniques have been used to assess toxicity in *V. cholerae* strain 569B and 12r (Ogawa) toxins by Latif (Latif, 1966). In this series of experiments only skin induration was studied. Latif showed that there was a very good correlation between induration and blueing effects produced by the same dose of toxin in guinea-pigs (Latif, 1966).

The use of skin induration as an index of toxin production appears to be a very sensitive method of gauging toxicity of crude filtrates. Although the time course is different from that seen with the skin blueing lesion size, it is also a good means of comparing the toxic abilities of different solutions. The blueing response

reaches its maximum size over a $1\frac{1}{2}$ to 8 hour interval, the lesion increasing from 2 to 8 mm, but the intensity of blueing continues to develop until 18 to 24 hours after the inoculation. Skin induration, however, caused by the same dose of crude filtrate shows a more gradual progression and the peak effect is seen at 18-24 hours. Figure 5 shows the relationship between induration and blueing and is adapted from Craig, 1971. It is in fact probably easier to compare the size of lesion at a given time than the intensity of the colour reaction in vivo.

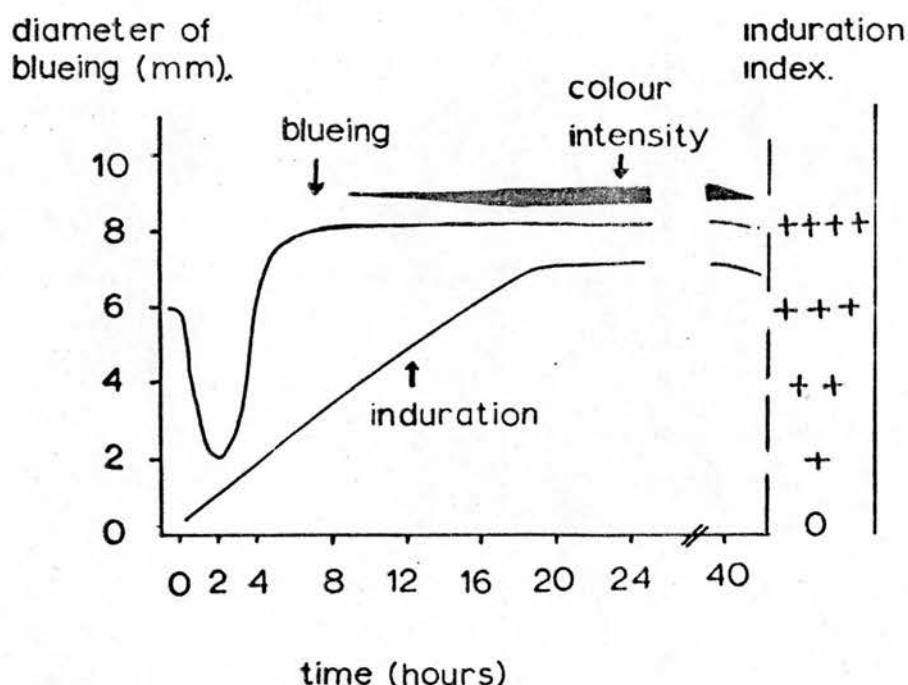


Figure 5.

Figure 5. The time course of enhanced capillary permeability and induration following intracutaneous injection of 0.1 ml of 1:20 diln of cholera stool filtrate in guinea-pigs (from Craig, 1971).

This technique was therefore used to indicate the presence of toxin activity in crude filtrates prepared from *V. cholerae* 12r (Ogawa).

Conclusions

(1) The production of enterotoxin by *V. cholerae* strain 12r (Ogawa) requires lower rather than higher temperatures under the conditions used to incubate live vibrios.

(2) The incorporation of neomycin or chlorhexidine did not appear to detract from the enterotoxicity of crude toxin filtrates.

The incorporation of neomycin (100 μ g/ml) into such filtrates adopted as a standard procedure to ensure bacterial sterility.

(3) Skin induration gives a sensitive index of toxin production. The size of the lesion is dependent on the dose of toxin filtrate used and shows a good correlation with the enterotoxic activity of the filtrate preparation under investigation.

Experiment 1

The Release of Prostaglandins in the rabbit ligated loop model of cholera.

The ileal, ligated loop in rabbits has been widely used in cholera research. It has the advantage of being a simple model to prepare, gives reproducible results (De et al, 1960; Jenkin & Rowley, 1959; Pierce & Wallace, 1973), and the fluid which accumulates in the loops resembles that secreted in human cholera. The model is analogous in some respects to certain tissue injuries e.g. burns and local inflammatory conditions in that fluid accumulation due to trauma occurs in a confined region. In both burn and inflammatory skin lesions prostaglandin release occurs (Jonsson & Hamberg, 1973; Greaves et al, 1971). It is possible therefore that in the ligated loop model, prostaglandin release into the pathologically produced fluid also occurs. This theory was tested.

Methods

Eleven rabbits, of both sexes, weighing 1.7 to 3.5 kg were deprived of food in the 24 hour period prior to the operation but were allowed free access to water. The animals were anaesthetized with pentobarbitone sodium, 40mg/kg, given by intravenous injection. The intestine was exposed at laparotomy and the terminal small intestine was exteriorized. A maximum of 8 loops each 10 to 15 cm long were created in the distal ileum by means of mersilk ligatures, the last loop reaching a point 15 cm proximal to the tip of the appendix.

Toxin solution, prepared either from V. cholerae 12 r and containing approximately 4.5 blueing units of activity per ml or purified V. cholerae 225B toxin containing 1 blueing units/15 µg

solid material and made up in stock solutions of 600-800 µg/ml in 0.9% saline, were used to challenge intestinal loops. Control loops, which were alternated with toxin-treated loops, were inoculated with either sterile control Difco-Bactopeptone medium or inactive toxin solution. Inactive toxin solutions were those solutions prepared by growing *V. cholerae* at 37°C and which contained no detectable skin induration activity in the animals receiving *V. cholerae* 12 r toxin. If *V. cholerae* 225B toxin was the secretory stimulant, then 0.9% saline injections were administered to the control segments. The volumes injected were 1.5 ml of the *V. cholerae* 12 r toxin and appropriate control solutions, and 0.1 ml of *V. cholerae* 225B toxin solution and normal saline.

After inoculation, the intestine was returned to the abdominal cavity and the incision was sutured. Animals were allowed to recover from the anaesthetic and returned to their cages. No food was given in the ensuing period but water was allowed. Animals were killed 8-12 hours after toxin challenge (5 animals) and 22-24 hours after toxin administration (6 animals). The fluid which had accumulated in the toxin loops was collected on ice and either extracted immediately or stored at -15°C until extraction could be carried out. The appearance of the intestinal wall was noted and the presence of any haemorrhagic necrosis was recorded.

Extraction of Samples

Standard techniques based on those described by Bergström and Samuelsson (1963) and Horton and Main (1967) were employed. Two methods (Method A and Method B) were used to extract prostaglandin-like material from the fluid samples. The procedures are outlined in Figures 6a and 6b.

Figure 6(a). The Extraction of Prostaglandins from Fluid Samples obtained from cholera toxin-challenged ligated, rabbit intestinal loops.

Method A

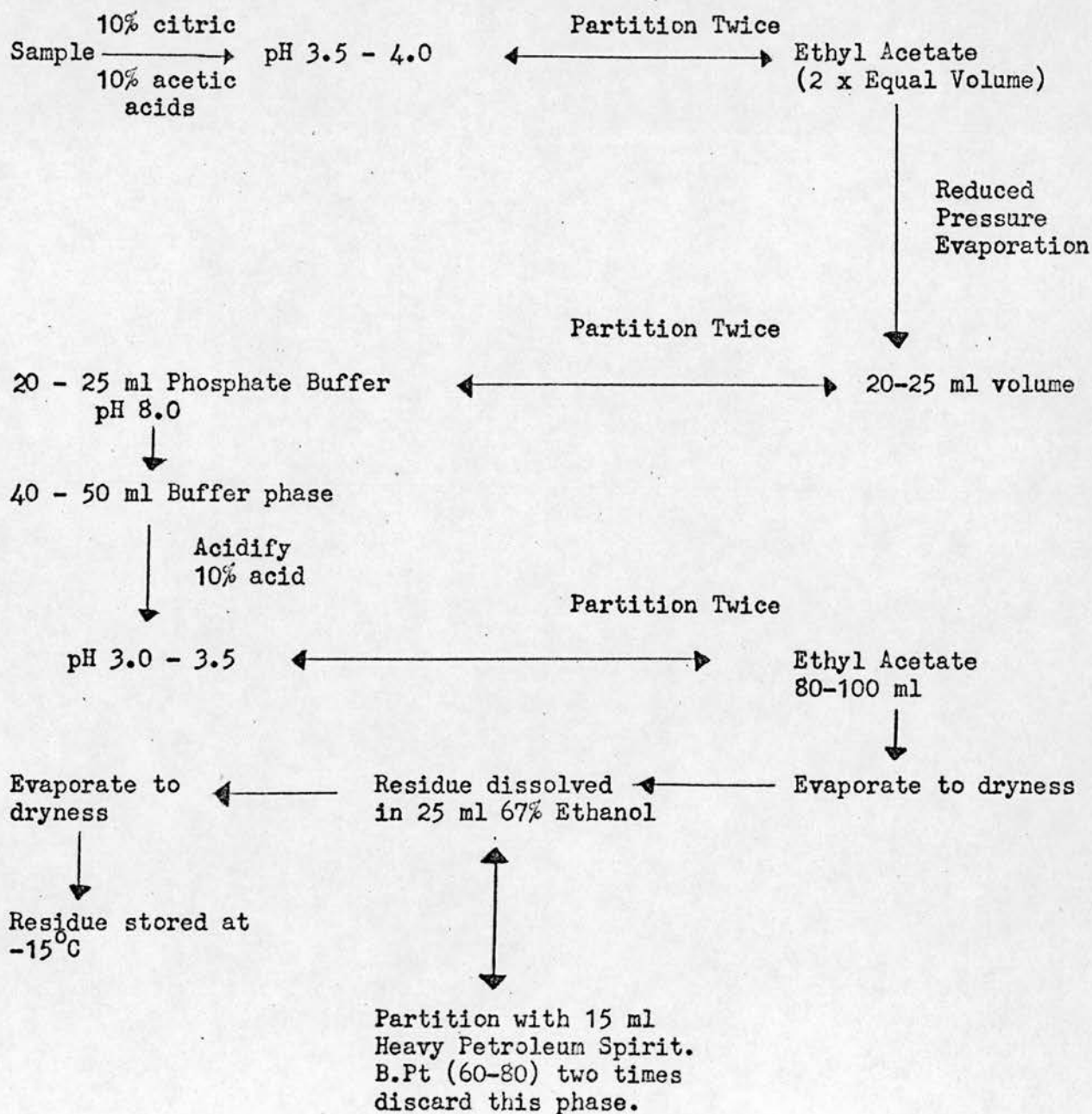
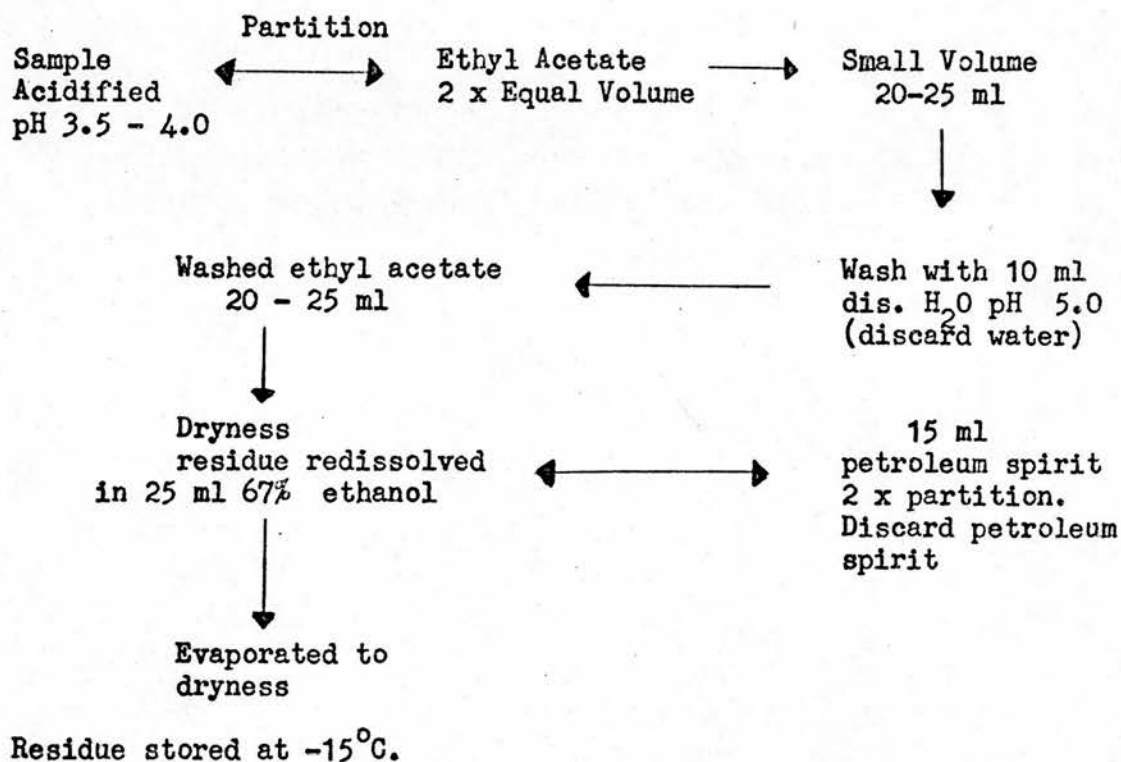


Figure 6(b)

Method B



Method A

Samples were acidified to pH 3.5 to pH 4.0 using either 10% citric acid or 10% acetic acid, after the volume had been measured. The acidified fluid was then partitioned twice with two times an equal volume of redistilled ethyl acetate and the organic phases were pooled, evaporated to a small volume (20-25 ml) and then partitioned with twice equal volumes of phosphate buffer, pH 8.0. This phosphate buffer partition was performed twice. The buffer fractions were combined and acidified to pH 3.0 to 3.5 using 10% acetic or citric acid. A second ethyl acetate partition followed; again using ^{two times an} ~~twice~~ equal volumes and two partitioning steps. The inorganic phase was discarded and the ethyl acetate fractions were combined, and evaporated to dryness under reduced pressure. The residue was dissolved in 25 ml of 67% ethanol and shaken with 15 ml heavy petroleum spirit. This petroleum phase was discarded and the ethanolic material treated similarly with a second 15 ml volume of petroleum spirit. The ethanol solution was then evaporated to dryness and desiccated. The residue was stored at -15°C prior to silicic acid chromatography or thin layer chromatography purification and separation of the prostaglandin-containing extract.

Method B

Samples were extracted into ethyl acetate as described in Method A. The ethyl acetate fraction was evaporated to a small volume (20-25 ml) and washed with 10 ml distilled water (pH less than 5.0). The washed ethyl acetate was evaporated to dryness under reduced pressure and the water phase was discarded. The residue was dissolved in 67% ethanol (25 ml) and the extraction of prostaglandins and prostaglandin-like material was completed as in Method A.

The results obtained by Methods A and B were not significantly different but the latter method had the advantage of being less time consuming.

Silicic Acid Chromatography:

The use of this technique to separate the different series of prostaglandin compounds was first described by ~~Bergstrom and~~ Samuelsson (1963).

Silicic acid 4.5 g SIL-R, 100-300 mesh or Bio-Rad SIL A, 100-200 mesh Batch 6910, was weighed out, dried and activated by heating at 100°C for 1 hour. After cooling the silicic acid, a slurry was made by adding petroleum spirit and this was then poured into a 10 cm long glass column 1 cm in diameter. The column was washed with 10% ethyl acetate in toluene. The solvent system used varied with the batch of silicic used: in the case of the Sigma silicic acid an ethyl acetate-toluene solvent system was found to be satisfactory but when Bio-Rad silicic acid was used, the addition of methanol was required. Increasing percentages of ethyl acetate in toluene were used to elute prostaglandins of the A and E series. The systems used to elute the PGA and PGE-like material in the fluid samples are outlined in Table 5. Poyser (1971) had shown that PGE compounds were eluted in 65% Methanolic Ethyl Acetate and 60% Ethyl Acetate-Toluene Mixtures with these batches of silicic acid. Prostaglandins of the A series were eluted in the 30% Ethyl Acetate-Toluene fractions. This was demonstrated by running standard columns using ³H-labelled PGA₁ and following the elution of the radioactive material by scintillation counting techniques.

The samples were dissolved in 10% Ethyl acetate in benzene and added dropwise to the top of the silicic acid column, using a Pasteur pipette. The flask was rinsed carefully with aliquots

Table 5

(a) Solvent Systems used to elute prostaglandins from Bio-Rad

SIL-A, 100-200 mesh Batch 6910, columns.

<u>Fraction Number</u>	<u>Volume (ml)</u>	<u>Solvent Content - Percentages</u>		
		<u>Methanol</u>	<u>Ethyl Acetate</u>	<u>Toluene</u>
1	40	0.05	19.95	80
2	100	0.10	29.90	70
3	20	0.10	29.90	70
4	40	0.25	39.75	60
5	130	0.60	64.4	35
6	20	0.60	64.4	35
7	130	2.5	77.5	20
9	40	100	-	-

(b) Solvent System used to elute prostaglandins from Sigma SIL-R,

100-300 mesh silicic acid.

<u>Fraction Number</u>	<u>Volume (ml)</u>	<u>Solvent content - Percentages</u>	
		<u>Ethyl Acetate</u>	<u>Toluene</u>
1	40	30	70
2	40	40	60
3	40	60	40
4	110	60	40
5	20	60	40
6	150	80	20
7	40	100	-
8	40	Methanol	-

of the first fraction and these were also added to the column. The columns were eluted at a rate of 1 ml/minute under negative pressure. Eluate fractions were collected and evaporated to dryness under reduced pressure. They were then stored at -15°C until biological assay could be performed. The original flask was also evaporated to dryness and stored similarly for biological assay of the residual content.

Silica Gel Thin Layer Chromatography

A detailed account of the preparation, loading and running of thin layer, silica gel, glass plates is given by Horton and Main (1967).

Grooved plates, 5 cm wide and 20 cm long were coated with a 0.25 mm thick layer of silica gel G (Merck). They were then dried and activated by heating at 100°C for 1 hour. The prepared plates were stored in a desiccator until required.

Samples which contained tritiated standard PGE_1 ($0.1 \mu\text{Ci}$) were ~~and~~ applied as a narrow band to the plates using an Agla micrometer syringe. The flasks were rinsed with 2 x 0.1 ml methanol and these fractions were also applied to the plates.

Thin layer plates were developed in the AI solvent system of Green and Samuelsson (1964) and subsequently in the AVII solvent system (Green and Samuelsson, 1964). If, however, the extracts were highly pigmented, they were developed twice in the G.C.M. solvent system described by Hensby (1974). Before the second solvent run, the origin zone of the first run was carefully scraped off and the plates were run from a new base line.

Plates were scanned using a Panax Radio TLC plate scanner. The sensitivity was set at 100 mV and the chart speed was 12 cm per hour. The time constant was 10 seconds and the range 10-30 cps.

Zones corresponding to the regions occupied by the standard prostaglandins were scraped from the plates and eluted with 2 x 5 ml methanol. The methanol fractions were evaporated to dryness under reduced pressure and stored at -15°C until bioassay on the appropriate tissue could be carried out.

Biological Assay

(a) The Rat Fundal Strip. The PGE-containing fractions from silicic acid columns and the corresponding zones from thin layer plates were assayed on the rat fundal strip preparation as described by Vane (1957). Only one longitudinal cut was made in the fundus giving a shorter, broader strip of tissue. The preparation was suspended in a 5 ml organ bath in Tyrode's solution at 37°C and was gassed with oxygen. Contractions elicited by doses of the samples were compared quantitatively and qualitatively with responses to standard PGE_1 . Stocks of standard PGE_1 solution containing 100 ng/ml PGE_1 were prepared. Doses of 5 to 10 ng were injected, giving a final bath concentration of 1-2 ng/ml. A three minute dose cycle, with a drug contact time of 45 to 60 seconds was used for standard and sample doses. The samples were dissolved in 1 ml distilled water for assay on this preparation.

(b) Kitten Blood Pressure Assay

PGA samples from chromatographic procedures were dissolved in 1 ml 0.9% saline.

Kittens weighing 1.0 to 1.8 kg, of either sex, were anaesthetized with pentobarbitone sodium 40 mg/kg injected intraperitoneally. Anaesthesia was maintained by slow intravenous infusion of pentobarbitone sodium solution 6 mg/ml at rates of 0.05 to 10 ml per hour. Carotid blood pressure was recorded on a Grass Polygraph. Standard PGA_1 was injected as a bolus into

the femoral vein and was washed in with 0.2 ml 0.9% saline (Horton & Jones, 1969). Doses of standard PGA_1 were injected (stock solutions - 250 ng/ml and 500 ng/ml). A ten to twelve minute dose cycle was used and the response to a sample injection was compared to that of standard PGA_1 for vasodepressor activity. Preparations were only used to assay samples if they were sensitive to 30 - 50 ng standard PGA_1 .

Gas Chromatography-Mass Spectrometry

Samples remaining after biological assay were not sufficient to allow for gas chromatographic-mass spectrometric analyses to be performed.

Statistical Evaluation of Results

Statistical analysis of the samples was carried out. Using the student 't' test, the significance of the difference of the mean values obtained for the 8-12 hour and 22-24 hour PGE_1 and PGA_1 output, and the rates of output were assessed.

Results

(a) Animals killed at 8 to 12 hours after toxin administration

No haemorrhagic necrosis was found in the small intestine wall in animals killed 8 to 12 hours after toxin challenge when either crude or purified toxin preparations were used. Fluid had accumulated in the ligated toxin-challenged loops but marked distension of the gut was not observed. Estimates of the prostaglandin content of the fluid were made. In 5 animals the mean output of PGE_1 -like material was 1.1 ng/ml fluid secreted in the observation period. In the case of 3 of the 5 rabbits no detectable PGA_1 -like activity was contained in the fluid samples. The lower limits of detection in these experiments were 1.0, 0.5

and 0.5ng PGA_1 equivalents per millilitre of fluid secreted. In two samples PGA_1 -like material was present. The total activity contained in these was 0.6ng/ml and 0.4ng/ml respectively. The mean hourly rate of prostaglandin production was also determined. The rate of release of PGE_1 -like activity was 0.1ng/ml per hour while in the case of the PGA_1 fractions the release was 0.06ng/ml per hour using the limits of the assay as the output in the three experiments where no PGA_1 material appeared to be released.

(b) Animals killed at 22 to 24 hours after toxin administration

In all animals killed at 22 to 24 hours after toxin challenge, significant haemorrhagic necrosis of the intestinal wall in the loops inoculated with active toxin was observed. All of these loops were markedly distended with fluid. Traces of blood were present in some of these samples. The prostaglandin levels in the fluid aspirated in these experiments were determined by biological assay. The prostaglandin output by this group of animals was significantly greater than that observed in the 8 to 12 hour experimental group ($p < 1\%$). The mean output of PGE_1 -like material was 13.6 ng/ml and the corresponding PGA_1 output was 15.0 ng/ml. The mean hourly rates of prostaglandin release were also calculated. The rate of PGE_1 release was 0.59 ng/ml per hour and that of PGA_1 was 0.64 ng/ml per hour. These values are considerably greater than those obtained in the experiments of shorter duration. The difference in both the PGE and PGA release per hour are statistically significant at the 1% level in the case of the PGE samples and at the 0.1% level for the PGA samples. The rate of PGE and PGA production did not differ significantly from each other in the 22 to 24 hour group of rabbits.

The results of both groups of experiments are contained in Tables 6 and 7 and in figures 7 and 8.

Table 6. The output of prostaglandins into fluid secreted by the rabbit small intestine following challenge of the ligated ileal loop with cholera toxin.

(a) 8-12 hour output of Prostaglandin-like material

Experiment Number	Sample Volume (ml)	Percentage Recovery %	Duration (Hours)	Corrected Concentrations	
				PGE ₁ mean + S.E.M. ng/ml	PGA ₁ mean + S.E.M. ng/ml
1	60	60.0%	8	2.2	N.D. 1ng/ml
2	128	69.0%	10	0.5	N.D. 0.5ng/ml
3	112	59.0%	9.5	0.88	N.D. 0.5ng/ml
4	135	59.0%	12	1.05	0.6
5	100	59.0%	12	0.90	0.4
Mean Values		61.2%	10.3	1.11 ± 0.29	0.6 ± *

Ratio PGE₁/A₁ mean output

11 : 6

* The figure represents the mean of the two values obtained in experiments in which PGA₁ activity was detected plus those in which limit of detection values were obtained. In Table 6b the mean value was calculated from the experiments in which the recovery of exogenous prostaglandin was more than 40%.

(b) 22-24 hour output of Prostaglandin-like material

Experiment Number	Sample Volume (ml)	Percentage Recovery %	Duration (Hours)	Corrected Concentrations	
				PGE ₁ ng/ml	PGA ₁ ng/ml
1	32	10.2%	24	3.3	N.D. 1ng/ml
2	35	57.6%	24	4.9	13.5
3	37	45.0%	24	17.2	17.6
4	44	53.8%	24	10.8	19.6
5	35	54.4%	23	19.0	14.2
6	40	40.0%	22	16.3	10.5
Mean Values		50.2%	23.4	13.6 ± 5.8 mean + S.E.M. 0.9	15.0 ± 3.5 mean + S.E.M. 1.0
Ratio PGE : PGA output					

Table 7. The rate of prostaglandin production by the rabbit small intestine and release into fluid accumulated in the ligated ileal loop following cholera toxin challenge.

The hourly rate of PGE_1 and PGA_1 is estimated from the mean output in the 8-12 hour (5 animals) and 22-24 hour (6 animals) experiments tabulated in Table 4. The statistical significance of the difference between the results of the 8-12 hour and 22-24 hour samples is indicated.

Sample	PGE_1 Mean output ng/ml	PGE_1 hourly rate of release ng/ml/hr	PGA_1 mean output ng/ml	PGA_1 hourly rate of release ng/ml/hr	Number of observations to obtain mean
8-12 hour experiment	1.11 ± 0.3	0.1	0.6 ± 0.1	0.05	n = 5
22-24 hour experiment	13.6 ± 5.8	0.6	15.0 ± 3.5	0.7	n = 6
Difference in output between 8-12 and 22-24 hour values	12.5 ± 6.1	0.5	14.5 ± 3.6	0.65	
Student 't' test p. values	$p < 1\%$	$p < 1\%$	$p < 1\%$	$p < 0.1\%$	

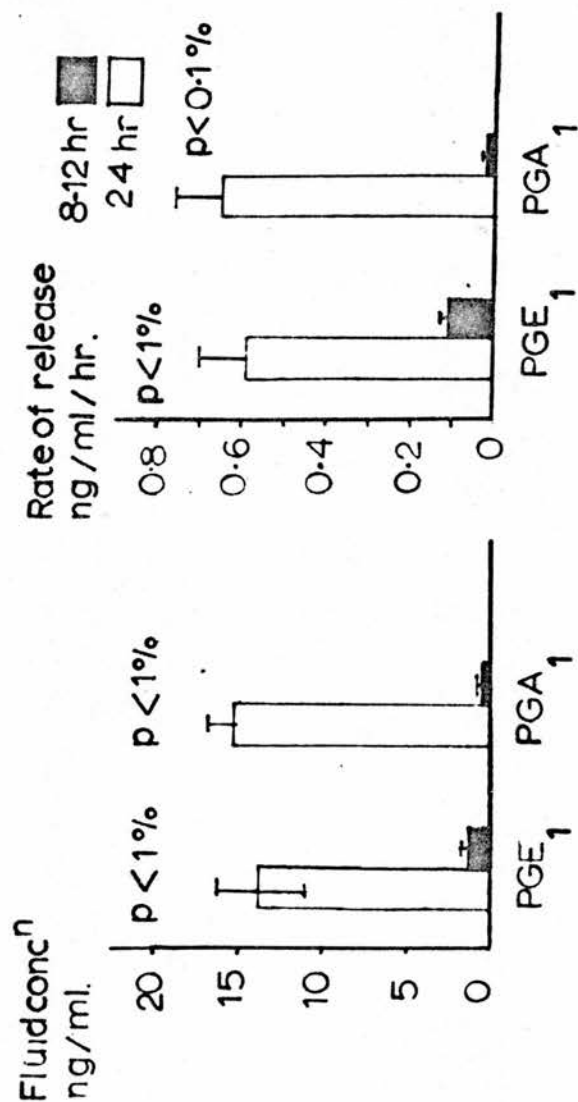


Figure 7. The comparison of prostaglandin release from rabbit intestine with time. Values represent Mean of 6 22-24 hr and 5 8-12 hr experiments \pm SEM.

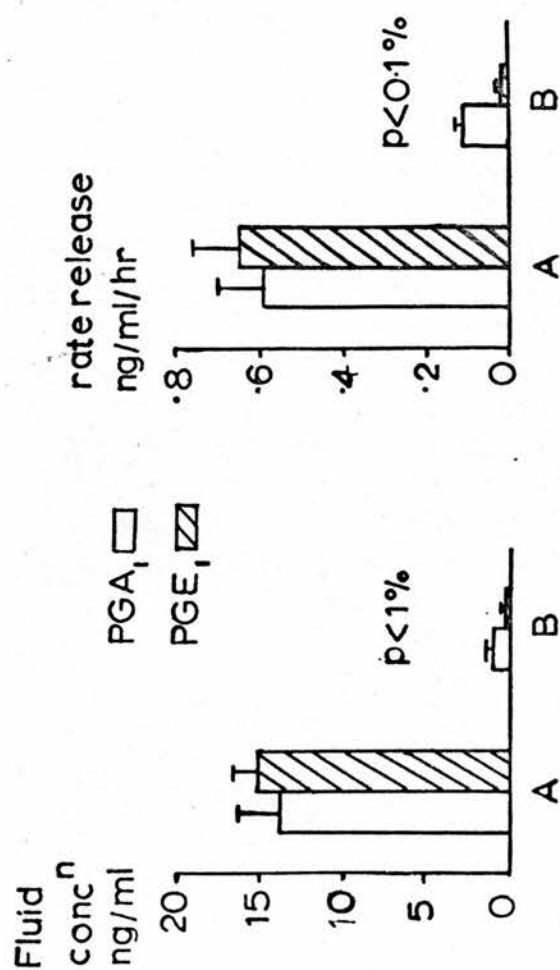


Figure 8. The release of prostaglandins in response to cholera toxin challenge. Group A at 22-24 hr Group B at 8-12 hr.

Discussion

Prostaglandins are released from the small intestine into fluid which accumulates in the ligated segment of the gut following cholera toxin administration. The release of prostaglandin-like activity is greater at 22 to 24 hours than at 8 to 12 hours after toxin challenge. This difference is statistically significant at the 1% level and corresponds to the onset of marked haemorrhagic necrosis and distension of the ligated intestine. The mean PGA_1 output at 8 to 12 hours after toxin administration appears to be approximately half of that observed for PGE_1 -like activity. The release being 1.1ng/ml for the latter and 0.6ng/ml in the case of the former (the lower limits of detection being used as estimates of the PGA content of fluid in which no actual activity could be detected). By 22 to 24 hours the release of PGA was equal if not greater than that of PGE_1 -like material. The amounts of PGE and PGA being released at this point being equivalent to 13.6 ± 5.8 ng/ml and 15.0 ± 3.5 ng/ml ^{Corrected values} respectively. The ratio for the mean output was shifted from 2:1 to 9:10 for PGE : PGA release at 8 to 12 hours and 22 to 24 hours respectively. Why such a shift should occur is not immediately apparent. There are several possible explanations which might account for the changes. At 12 hours the peak secretory activity has been achieved in the toxin challenged intestine. Thus it is feasible that the decrease of PGE release in favour of PGA is a reflection of the operation of some patho-physiological mechanism in the small intestine which is responsible for limiting fluid secretion. A second possibility is that the enhanced PGA content of the secreted fluid is an artefact and merely reflects the increased general release of PGE compounds due to tissue damage and distension of the intestinal wall. The augmented

PGE output with its subsequent dehydration to the corresponding PGA compound in the alkaline environment, such as is encountered in the lumen of the intestine, giving rise to the difference. Because the PGA present is more readily detected it only appears that there has been some alteration in the respective amounts of PGE and PGA released from the intestine after 22 to 24 hours have elapsed from the time of toxin administration. A third point must also be considered. The release of prostaglandin need not necessarily be limited to the intestinal tissue itself. It is possible that *V. cholerae* toxin itself or by interacting with the other microorganisms, causes prostaglandins to be released.

Conclusion

Prostaglandins are released into the fluid which accumulates in the ligated intestinal loop in response to cholera toxin challenge. The amounts present at 22 to 24 hours are significantly greater than those detected in fluid samples from animals killed at 8 to 12 hours after toxin administration. This increase coincides with the development of marked haemorrhagic necrosis and distension of the intestinal wall of cholera toxin-challenged loops.

Several questions are posed by these series of experiments.

- (1) What contribution does the crude toxin itself make to the fluid content of prostaglandins?
- (2) To what extent is the 8 to 12 hour output of prostaglandins a reflection of the enhanced secretory activity of the small intestine induced by cholera toxin? How much of the output is due to the onset of tissue damage and increasing pressure on the intestinal wall due to fluid accumulating in the loop?
- (3) Is the enhanced output observed in the 22 to 24 hour group of

rabbits attributable solely to the tissue damage and distension?

Could the enhanced output be indicative, even partly, of the operation of a pathophysiological mechanism which limits fluid production in the intestine challenged with cholera toxin?

Answers to these questions were sought in further experiments.

Experiment 1a

The prostaglandin content of Wyeth lot 002 crude cholera toxin

In experiments using impure cholera toxin it was possible that prostaglandin activity detected in the fluid secreted by the small intestine in response to cholera toxin challenge, might have originated from the toxin solution. In addition it was desirable to evaluate the relative contribution of toxin "prostaglandin" and intestinally produced prostaglandins to the total prostaglandin content of the intestinal secretions. Experiments were therefore undertaken to establish whether or not crude toxin solutions contain prostaglandin-like material or not. The toxin investigated was Wyeth lot 002 crude toxin. Previously Horton and Collee (unpublished observations) had been unable to detect any ^{prostaglandin-like} ~~such~~ activity in crude filtrates of toxin preparations obtained from V. cholerae strain, 12^r (Ogawa). Therefore it was not investigated.

Methods

In four experiments, the prostaglandin content of Wyeth 002 toxin was determined. One gram of crude toxin was weighed out and dissolved in 10 ml salt solution (Pierce et al, 1971). The pH was adjusted to pH 4.0 - 4.5 with 10% acetic acid. The following standard tritiated, high specific activity prostaglandins were added prior to extraction: PGE₂ (0.1 μ Ci : 49.6 ng cold PGE₂/ μ Ci), PGF_{2 α} (0.1 μ Ci: 36.5 cold ng/ μ Ci) and PGA₂ (0.1 μ Ci : 2.5 ng cold PGA₂/ μ Ci).

Extraction Procedure

The acidified samples were partitioned with 30 ml ethyl acetate twice and the organic phases were pooled, washed with 10 ml distilled water (pH \leq 5.0) and evaporated to dryness under reduced pressure. The residue was redissolved in 25 ml 67% ethanol and shaken with 10 ml heavy petroleum spirit. This partition was

performed twice. The petroleum fraction was discarded and the 67% ethanol phase was evaporated to dryness as before and desiccated. It was stored at -15°C prior to thin layer chromatography.

Thin layer chromatography

Samples were dissolved in 0.2 ml methanol and applied as a narrow band on to 0.25 mm thick Merck Silica Gel G prepared plates 5 x 20 cm in size. The plates were developed in a multi-run chromatographic development system. The initial run was performed using the F VI solvent system of Andersen (Andersen, 1969) while the second development of the plates was carried out in the GCM solvent system as described by Hensby (Hensby, 1974). The solvent front on both runs was 15 cm. from the origin.

The plates were scanned using a PANAX radio thin-layer chromatographic plate scanner. The sensitivity setting was 100 mV for the chart recorder, and 30 counts per second range and a time constant of 10 seconds on the detector. The chart speed was 120 mm per hour. A typical scan is shown in figure 9.

From the scan picture obtained it was possible to mark out the PGE, F and A containing zones of the plates and scrape them off the plates into test tubes containing methanol (5 ml). The methanolic fractions were centrifuged for 10 minutes using a bench centrifuge (at approximately 750g) and the supernatant was decanted off. The residue was resuspended in a further 5 ml methanol and recentrifuged. The methanol was collected and pooled with the initial methanol eluates. The combined eluates were evaporated to dryness and redissolved in 1 ml methanol. An aliquot (0.1 ml) was removed for recovery estimations while the remainder was evaporated to dryness and stored at -15°C until biological assay could be performed.

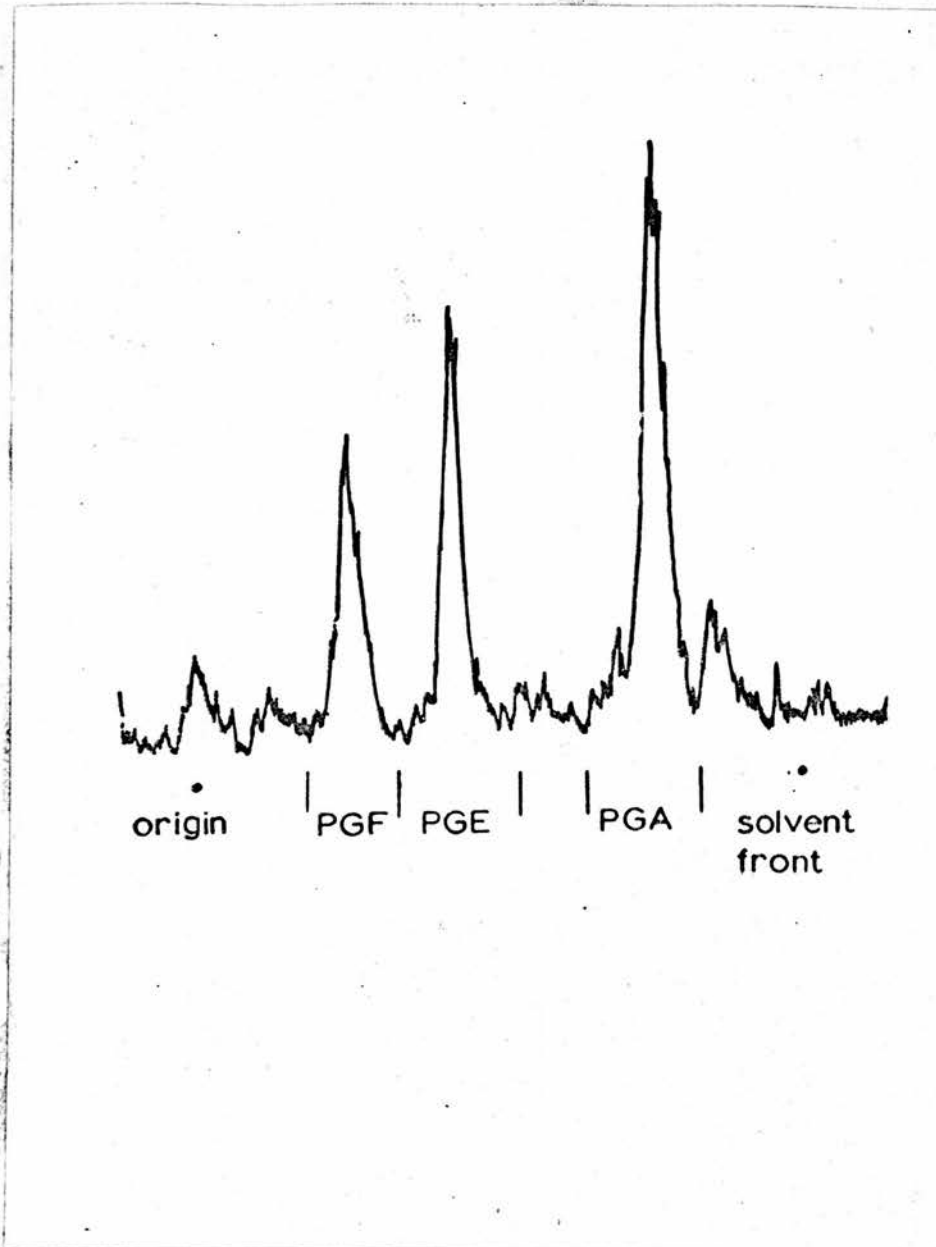


Figure 9. Thin-layer radiochromatographic scan of an extracted sample of Wyeth lot 002 toxin, containing approximately 0.1 μCi of standard tritiated prostaglandins, PGE_2 , PGA_2 and $\text{PGF}_{2\alpha}$ following development of the plate in FVI and GCM solvent systems.

Recovery Estimations

The percentage recovery of exogenous prostaglandin was determined by using liquid scintillation counting techniques. The 0.1 ml samples removed after thin layer chromatography were counted in 10 ml toluene ethoxyethanol based scintillant (Toluene-ethoxyethanol-DMFPOPOP-PP0 (2000,400,0.22,8.52, v/v/w/w). Counting efficiency was 35-40%. Correction for quenching was made by the use of the external standard channels ratio method and a quench calibration curve constructed for the scintillation mixture.

Biological Assay

(a) Rat Fundal Strip

Samples containing PGE and PGF-like activity were assayed in terms of standard PGE₂ and PGF_{2α}. Stock solutions containing 100 ng/ml of these standard prostaglandins were prepared. The rat fundal strip was suspended in Tyrode's solution at 37°C and gased with oxygen. The responses of the tissue to standard PGE₂ and PGF_{2α} were recorded and the responses to the samples was compared qualitatively and quantitatively with those of the appropriate standard. The preparation was sensitive to 0.5 ng/ml PGE₂ and 3 ng/ml PGF_{2α}. With PGE₂ a dose cycle of 3 minutes' duration and a drug contact time of 45-60 seconds was employed. In the case of PGF_{2α}, standard and samples, a longer cycle was required. A 3½ minute cycle and 60-90 minute drug contact time were used.

(b) Kitten Blood Pressure

The kitten blood pressure preparation was used to estimate the PGA content of cholera toxin extracts. Standard PGA₁ was given as a single intravenous injection (0.2 ml stock solution) and was washed in with 0.9% saline (0.2 ml). Stock solutions containing 100-500 ng

PGA₁ were prepared. A dose cycle of 8 to 12 minutes was required. The preparation was sensitive to 30 ng PGA₁/kg. PGA eluates from thin layer plates were redissolved in 0.5 ml or 1.0 ml 0.9% saline for injection. The vasodepressor activity of aliquots of these samples was assessed in terms of standard PGA₁.

Results

No PGE₂ or PGF_{2α} activity was detected in toxin extracts when assayed on the rat fundal strip. The lower limit of detection of PGE₂ indicated that the samples contained less than 12.5 ng PGE₂-like activity and less than 25 ng PGF_{2α}-like material per gram crude toxin. No PGA₁-like material was found. The maximum amount which could have been present and not detected by biological methods was less than 90 ng PGA₁/g crude toxin.

Discussion

The absence of prostaglandin-like activity in crude cholera toxin preparations from two different strains of *V. cholerae*, namely *V. cholerae* 569B used to prepare Wyeth 002 toxin, as reported in this experiment, and from *V. cholerae* strain 12r (Ogawa) investigated by Horton and Collee (unpublished observations) would seem to indicate that any prostaglandin contained in the fluid secreted in response to cholera toxin challenge is unlikely to originate from the crude toxin itself. Purified cholera toxin and crystalline toxin contain less than 1% lipid material and protein moieties (Finkelstein & Lo Spalluto, 1969; 1970; Finkelstein & Lo Spalluto, 1972; Finkelstein, 1972). Gas liquid chromatographic analysis of the lipid content of *V. cholerae* toxin has shown the presence of saturated and unsaturated fatty acids (Kaur et al, 1970). Kaur,

Konig, Martin and Burrows were investigating enterotoxin from *V. cholerae* 569B and found only C_{14} , C_{16} , C_{18} fatty acids in this preparation. Prostaglandin precursors are polyunsaturated C_{20} fatty acids. Removal of the lipid material did not result in a loss of enterotoxic activity of the toxin preparations (Kaur et al, 1970). This evidence, together with the results cited in this experiment do not favour a contribution to the prostaglandin activity of fluid samples from the toxins themselves and suggests that all prostaglandins are likely to be of intestinal origin.

Conclusion

Crude cholera toxin does not contain prostaglandins. Any prostaglandin found in fluid accumulating in the small intestine subsequent to exposure to cholera toxin is very probably of intestinal origin.

Experiment 2

The effect of indomethacin on fluid secretion and prostaglandin release in the ligated ileal loop model of experimental cholera in rabbits

There are several possible approaches to the problem of determining the role of prostaglandins in the ligated ileal loop model of cholera. One is to study the effect of prostaglandin synthesis inhibitors on fluid secretion following cholera toxin challenge.

Indomethacin, which inhibits the incorporation of molecular oxygen into prostaglandin precursors in the bio-synthetic pathway (Tomlinson et al, 1972) and thus suppresses the formation of prostaglandins, was used as a tool to investigate the function of these hormones in the secretory processes in the ligated ileal loop model of experimental cholera in rabbits.

Methods

Nine rabbits, weighing 1.5 to 3.3 kg were used in this study. The animals were New Zealand White stock of both sexes. Six animals were pretreated with the drug, indomethacin, while three served as untreated controls. Three different dose regimes were employed. The experimental procedure for loop preparation and handling of the animals during the pre-and post operative period was exactly as described in the previous experiment. (Experiment 1, Methods Section: Experimental Model).

Drug Treatment

Group A. There were two animals in this experimental group. The rabbits each received a single injection of 10 mg/kg indomethacin intravenously 40 minutes prior to toxin challenge. Two further doses of the drug (each of 10 mg/kg), were given to

the rabbits at two to four hourly intervals during the eight hour period following toxin challenge. Thus the total dose received by the rabbits comprising Group A was 30 mg/kg indomethacin.

Group B. The three rabbits in this group were pretreated with indomethacin given in a dose regime which was commenced three days prior to toxin challenge. Each animal was given a loading dose of 80 mg/kg indomethacin subcutaneously injected on the afternoon of the first day of treatment. On the next two days the rabbits received doses of 40 mg/kg indomethacin per day divided into two doses, each of 20 mg/kg, which were injected in the morning and in the evening respectively. On day four, the day of the experiment, animals were given the usual injection of indomethacin (20 mg/kg) prior to toxin challenge and were given further injections of 40 mg/kg amounts of the drug at four and eight hours after toxin challenge. The total dose given to each rabbit was 260 mg per kg indomethacin.

Group C. Two rabbits were each injected with a 20 mg/kg intravenous dose of indomethacin forty minutes prior to inoculating the intestinal loops with toxin. This dose, 20 mg/kg, was then administered at four hours and again at 8 hours after toxin challenge. These animals received a total of 60 mg/kg indomethacin.

Group D. The animals in group D were not given indomethacin injections. They were only challenged with cholera toxin as in Experiment 1 rabbits.

The animals in Groups, B, C and D were killed at 8-12 hours after toxin challenge. The two rabbits in Group A were allowed to survive for 24 hours.

Fluid was collected from the loops as described in Experiment 1 and either subjected to prostaglandin extraction procedures

immediately or were stored at -15°C until extraction could be carried out.

Extraction, Purification and Separation of Prostaglandins

Samples from Groups B, C and 2 animals in Group D were pooled. Fluid from Group A animals and the third rabbit in Group D were treated separately. Samples were extracted by Method B outlined in the Methods Section of Experiment 1, and Figure 6b. Separation of the PGE and PGA content of the samples was achieved by thin layer chromatography using the A I and A VII solvent systems of Gréen and Samuelsson (Gréen & Samuelsson, 1964) and described in the previous experiment (Methods Section: thin-layer chromatography). Samples were scraped from the plates and eluted from the silica Gel as described in the previous experiment and stored at -15°C until biological assay could be performed.

Biological Assay

The estimation of the PGE and PGA-like activity contained in the thin layer eluates was performed on the rat fundal strip and the kitten blood pressure preparations as described previously (Experiment 1, Methods Section: Biological Assay).

Results

The effect of indomethacin pretreatment on fluid production and prostaglandin release in the ligated loop model are summarized in Table 8 and in Figure 10.

Group A

These 2 rabbits were allowed to survive for 24 hours. The fluid output was 60 ml and 38 ml in 4 and 3 toxin treated loops respectively. This was a comparable output to animals receiving no indomethacin (Results Section, Experiment 1). The prostaglandin

output was diminished compared to prostaglandin concentrations detected in the 24 hour fluid samples in Experiment 1 animals which received no drug treatment. The PGE_1 concentration in the two Group A rabbits was 3.0 ng and 1.7 ng/ml. No PGA_1 activity was detected. The limit of the assay was 60 ng PGA_1 for each of the samples. Thus there was less than 1 ng/ml and less than 1.6 ng/ml in the 60 ml and 38 ml samples respectively. In Experiment 1, the mean PGE output in the 24 hour fluid was 13.6 ng/ml and the PGA concentration was 15.0 ng/ml. No haemorrhagic necrosis was observed in the intestine of these animals.

Group B

The fluid from these three animals had been pooled and extracted, purified and assayed as a single sample. The PGE output was 0.7 ng/ml but no PGA_1 activity could be detected. The limit of assay was 90 ng PGA_1 for the total sample. Thus the concentration was less than 0.6 ng/ml in the PGA fraction of the pooled fluid.

Group C

Fluid collected from the two animals in this group was also pooled and treated as one sample. The volumes were 45 ml and 31 ml respectively from 3 loops in each animal. The PGE_1 concentration in the pooled material was 9.65 ng/ml. Again no PGA_1 was detected. The limit of sensitivity of the assay method for PGA_1 was 90 ng PGA_1 for the total sample. Thus the PGA_1 concentration in the choleraic fluid was less than 1.2 ng/ml.

Group D

The control animals' fluid was pooled in the case of two of the rabbits. The fluid output was 54 ml and 83 ml in 4 loops and 5 loops respectively. In the third animal a total of 44 ml

was recovered from 3 toxin challenged loops. The fluid was not pooled with the combined samples of the other two rabbits. The mean output of PGE_1 was 1.8 ng/ml in the 9 hour period. No PGA_1 activity was found. The limit of sensitivity of the PGA_1 assay was 90 ng PGA_1 total for each of the two samples. Thus the concentration of PGA_1 was less than 0.7 ng/ml and less than 2 ng/ml in the pooled control and unpooled control samples respectively. The mean output was higher than that detected in Experiment 1 control 8-12 hour animals' fluid but the range of output in these animals was quite large and thus the discrepancy in mean figures is probably a reflection of the fewer numbers of animals used in this experiment.

Discussion

Indomethacin treatment in the doses described is capable of reducing the prostaglandin output into fluid which accumulates in the ligated ileal loop of the rabbit intestine following cholera toxin challenge. The reduction was more marked in the 24 hour animals than in the 8-10 hour group of animals. This, together with the notable absence of haemorrhagic necrosis of the intestinal wall of the 22 to 24 hour group of ^{indomethacin treated} rabbits, suggests that the main contribution to the prostaglandin release may in fact be tissue destruction. The fluid output was not suppressed. The residual prostaglandin levels may therefore be attributable to distension of the intestinal loops by this accumulation of fluid. It is not however possible to exclude the possibility that this remaining prostaglandin output may be involved in the secretory processes initiated by cholera toxin.

Further experiments, in which the element of distension is

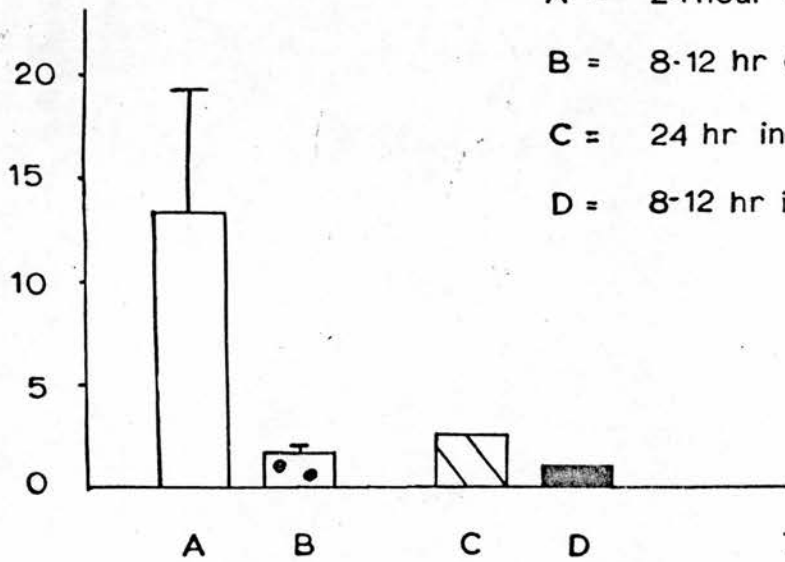
Table 8

The Effect of Indomethacin pretreatment on Prostaglandin Output and Fluid Production in the Rabbit Ligated Ileal Loop Model of Cholera.

Duration hrs.	Group	No. of Rabbits	No. of Loops	Volume	Total PGE ₁ ng	Concentration PGE ₁ ng/ml	Total PGA ₁	Concentration PGA ₁ ng/ml
24	A	2	a. 4	60	180	3.0	60ng	<1.0 ng/ml
			b. 3	38	66	1.7	60ng	<1.6 ng/ml
			MEAN			2.4 ng/ml		<1.3 ng/ml
10	B	3	a. 4	48	100	0.7 ng/ml	90ng	<0.6 ng/ml
9.6			b. 3	44				
9.75			c. 4	55				
9	C	2	a. 3	45	50	0.7 ng/ml	90ng	<1.2 ng/ml
9.5			b. 3	31				
9	D	3	a. 4	54	200	1.4	90	<0.7 ng/ml
9			b. 6	83				
8.75			c. 4	44	100	2.2	90	<2.2 ng/ml
			MEAN			1.8 ng/ml		<1.5 ng/ml

Figure 10.

PGE concⁿ
ng/ml



KEY

- A = 24 hour control
B = 8-12 hr control
C = 24 hr indomethacin
D = 8-12 hr indomethacin

Figure 10. The release of PGE-like compounds from rabbit ligated intestinal loops following indomethacin pretreatment. Values for Column A are taken from Experiment 1, Table 7; those for groups B, C and D from Table 8. Values represent mean results \pm S.E.M. where appropriate.

also absent are required in order to determine whether the residual release is due to stretching of the epithelial cells and intestinal wall or is essential to fluid secretion in response to cholera toxin.

Conclusion

Indomethacin in the dose regimes outlined caused a marked reduction in prostaglandin output into fluid accumulating in the ligated loops of animals killed at 22-24 hours. The output at 8-12 hours was also reduced. It is not clear whether the residual prostaglandin release is essential to the secretory process or is a reflection of the loop distension. Further experiments are required to clarify this point.

Experiment 3

The release of prostaglandins from the luminally perfused rabbit small intestine, in response to cholera toxin challenge.

The use of ligated intestinal loop models of cholera had two disadvantages. The first of these was the inability to compare the output of prostaglandins by non-toxin treated intestine as no secretion of fluid occurred in the ligated control loops. The second disadvantage was the presence of other factors likely to lead to prostaglandin release, in themselves. Distension of the tissue stimulates the production and release of prostaglandins (Poyser, 1971) and traumatization of tissue also causes prostaglandin release (Piper & Vane, 1971).

In experiments in which the ligated loop model was employed to study fluid secretion induced by *V. cholerae* toxin, it was not possible to dissociate the prostaglandin release due to distension of the intestine from that due to the effect of the toxin on the secretory processes. By using a luminally perfused intestinal loop model it was possible to eliminate the prostaglandin release arising from the distension of the intestine. It was intended to investigate the release of prostaglandins into the luminal perfusate from the toxin-challenged and control loops and so define the role of prostaglandins in the secretion of fluid stimulated by the toxin.

Methods

Seven rabbits, New Zealand White and Brown and Black Lop animals of both sexes were used in this series of experiments. The rabbits, weighing between 1.8 and 3.9 kg, were anaesthetised with pentobarbitone sodium, 40 mg/kg administered by intravenous injection. Anaesthesia was maintained by the infusion of pentobarbitone sodium solution, 5 mg/ml,

via the external jugular vein. The abdomen was incised and the small intestine was exteriorized and two loops were tied off in the terminal small intestine by means of Mersilk ligatures. These loops were longer than those used in the ligated loop model, being of 25 to 35 cm in length. This was because it was more practical to perfuse only two loops in any one animal. An unperfused segment of intestine 4 to 6 cm long was left between the two perfused sections of gut. The test (toxin challenged) loop and the control (non-toxin treated) loop were cannulated at their upper and lower ends and fluid was circulated through the lumen of these loops at a rate of 1.5 to 2.0 ml per minute. The perfusate was 0.9% saline warmed to 37°C prior to use. Cholera toxin either 0.1 ml purified 225B toxin or 1.5 ml crude *V. cholera* 12r/T30/N100 (Section 1, Part 1, Methods section: Preparation of cholera toxin), was introduced into the lumen of the test loops and the inflow and outflow cannulae were clamped off from their respective reservoirs for one hour. Control loops were inoculated with an equal volume of 0.9% saline (225B toxin treated animals) or control sterile Difco BactoPeptone water medium or inactive toxin solution prepared by incubating the *V. cholera* strain at 37°C and allowing the supernatant to stand at room temperature for several days. This resulted in the loss of any residual toxic activity in the fluid. The loops receiving these last-mentioned control fluids were those in animals being challenged with toxin prepared from *V. cholerae* strain 12r. Control loops were also clamped off in a similar manner to the toxin-treated loops. After the hour-long incubation period, perfusion of the lumen was begun and was continued for a period of 4½ to 5½ hours. The fluid was collected on ice and extraction of the samples was either carried out immediately or the material was stored at -15°C until

the extraction could be performed.

Treatment of Fluid Samples

The perfusate from toxin and control loops of two rabbits was pooled separately in six of the seven animals used in this series of experiments. Thus there were three samples for toxin and control loops from these rabbits. The material obtained from the seventh animal was treated independently and although subjected to exactly the same extraction, purification and separation techniques as the pooled samples, contained standard tritiated prostaglandins in tracer amounts to enable estimates of the recovery of endogenous prostaglandin-like material in the perfusate to be made. The amounts of $^3\text{H-PGE}_1$ and $^3\text{H-PGA}_1$ added to the unpooled material were $0.1 \mu\text{Ci}$ of each but the amount of non-radioactive prostaglandin present was 150 ng in the case of the PGE_1 and 100ng PGA_1 .

Extraction, Purification and Separation of Prostaglandins

The technique used to extract, purify and separate the prostaglandin-like activity in the intestinal fluid have been described previously (Section 1, Part 2: Experiment 2).

Recovery of Exogenous Prostaglandin Activity

Aliquots of the PGE_1 and PGA_1 fractions of the unpooled sample (0.1 ml) were taken after dissolving the residue obtained following thin-layer chromatography in 1 ml methanol. The amount of radioactivity contained in these aliquots was then determined by scintillation counting. The 0.1 ml samples were added to 10 ml toluene scintillant fluid and the tritium content was measured using a Nuclear Chicago Scintillation Counter. The remainder of the sample was evaporated to dryness and subjected to biological assay as were the other samples.

Biological Assay

The estimation of the prostaglandin content of the perfusate was made by assaying the PGE and PGA-containing zones on the rat fundal strip and the kitten blood pressure preparation respectively, as described in Experiment 1 (Section 1, Part:2.Experiment 1).

Results

The prostaglandin-like activity in the samples was estimated in terms of the PGE₁ and PGA₁ equivalents for toxin and control loops. The results are summarized in Table 8.

(a) The unpooled sample

The recovery of prostaglandin activity in the sample was 67% and 50.8% for the control and the toxin-treated loops respectively. Fluid from the toxin challenged loop was contaminated with blood and this is very probably responsible for the much lower recovery of exogenous prostaglandin. The total PGE₁-like content of the fluid from the control loop was 130ng PGE₁ equivalents and that of PGA₁ was 115ng PGA₁ equivalents as determined by biological assay. The fluid obtained from the toxin-treated loop contained very different amounts of these prostaglandins. The PGE₁-like activity amounted to only 50 ng whereas the amount of PGA₁-like material present in the extracted and purified PGA₁ zone was estimated to be 190 ng. The presence of blood in the perfusate may account for this result.

(b) Pooled Samples

No prostaglandin-like activity was detected in any of the samples. The limits of the assays were 20 ng and 75 ng for the PGE₁ and PGA₁ content of these samples respectively.

Table 9. The release of prostaglandin-like activity in toxin-challenged and control intestinal loops luminally perfused with salt solutions.

Experiment Number	Sample Toxin/control	Volume (ml)	PGE ₁ (ng total)	PGA ₁ (ng total)
1 Unpooled sample	Toxin	112 ml	50ng (150 added)	190 (100 added)
	Control	78 ml	130ng (150 added)	115ng (100 added)
2 Pooled a, b	Toxin a Toxin b	177 ml	< 20ng	< 75ng
	Control a Control b	134 ml	< 20 ng	< 75ng
3 Pooled c, d	Toxin c Toxin d	260 ml	< 20 ng	< 75ng
	Control c Control d	274 ml	< 20 ng	< 75ng
4 Pooled e, f	Toxin e Toxin f	206 ml	< 20 ng	< 75ng
	Control e Control f	193 ml	< 20 ng	< 75ng

Discussion

No prostaglandin-like activity was detected in the purified extracts of luminal perfusion fluid collected from rabbit intestinal loops challenged with cholera toxin. No prostaglandin-like material was present in control perfusates either. The lumen of the intestinal loops was perfused for $4\frac{1}{2}$ to $5\frac{1}{2}$ hours. The small intestine of other species has been found to contain prostaglandin dehydrogenase activity (Änggård, Larsson & Samuelsson, 1971). Parkinson and Schneider (1969) have demonstrated that approximately 7 to 12% of a lumenally perfused dose of (1 - ^{14}C) PGE_1 is removed from the perfusion fluid in one hour by the intestine. Thus it was possible that some inactivation of released prostaglandin did occur. Prostaglandin output would have to be continuously maintained if cholera toxin-induced diarrhoea was dependent on prostaglandin compounds (see Pierce et al, 1971). Each sample assayed was prepared from the intestinal perfusate of two separate loop perfusion experiments. PGE_1 -like content was estimated by the rat fundal strip technique. This preparation could detect 5 ng standard PGE_1 . The PGE_1 -like material in these samples ranged from less than 0.06 ng to less than 0.16 ng per ml perfusion solution. The PGA_1 -like activity was assessed on the kitten blood pressure preparation, which was sensitive to 25 ng standard PGA_1 . It was observed that the samples contained less than 0.2 ng to less than 0.6 ng PGA_1 -like activity per ml perfusate. These values represent the total output of PGE_1 -like and PGA_1 -like activity during the entire perfusion period. Even allowing for metabolism to the extent of 20%, it would appear that negligible amounts of prostaglandin-like material were being released. Burn blister fluid has

been found to contain approximately 1.4 ng PGE₁ equivalents (Arturson, Hamberg, Jonsson, 1973) per ml fluid. Greaves and his colleagues detected prostaglandin E-like activity in perfusates of inflammatory lesions in skin. The PGE₁-like material was in the region of 1ng PGE₁ equivalents per millilitre of perfusates. This contrasted to levels below 0.2 ng/ml perfusate in control samples (Greaves et al, 1971). The results would therefore seem to indicate that cholera toxin did not induce prostaglandin production. In the absence of more sensitive assay techniques it was not however possible to eliminate prostaglandin involvement in the fluid production completely. Exogenous administration of prostaglandin in large amounts does stimulate fluid secretion (Pierce et al, 1971). However sensitive the assay might be, it is unlikely that one could be absolutely certain that prostaglandin-like compounds did not mediate, at least in part, the effects of cholera toxin with negative findings in studies of similar nature to these experiments.

Conclusion

It is unlikely that prostaglandins are the mediators of the effects of cholera toxin in the intestinal mucosa. The output of prostaglandin E-like and A-like material into intestinal perfusates was negligible and was not detectable by biological assay.

Experiment 4

The effect of local administration of indomethacin on the secretory response of the perfused rabbit ileal loop to cholera toxin.

In the preceding experiment no PGE_1 -like nor PGA_1 -like material could be detected in extracted purified samples of intestinal perfusate following cholera toxin administration. It was considered unlikely that prostaglandins were involved in the pathogenesis of fluid loss but taken by itself, the results of these experiments were not conclusive enough to exclude prostaglandin participation entirely. Therefore, it was decided to investigate the effect of indomethacin on fluid production in the perfused loop model in rabbits subsequent to toxin challenge. Local administration of indomethacin to the spleen (Ferreira & Moncada, 1971; Ferreira, Moncada & Vane, 1971) caused a significant reduction or total inhibition of prostaglandin release in response to hormonal and nervous stimulation. Indomethacin is not selectively concentrated by the intestine of several species examined (Hucker, Zacchei, Cox, Brodie and Cantwell, 1966) and thus intestinal levels of the drug following systemic administration are dependent on the total dose administered. Accepting various reservations, it was hoped that local administration would ensure that high mucosal epithelial concentrations of the drug were achieved. If prostaglandins were vital to the secretion of fluid, then by local administration of indomethacin in high concentration, it should have been possible to reduce the fluid output observed in response to cholera toxin challenge.

Methods

Seven New Zealand White rabbits, weighing between 1.8 and 3.8 kg were anaesthetized with pentobarbitone sodium, 40mg/kg, injected intravenously. Anaesthesia was maintained by infusing a

solution of pentobarbitone 6 mg/ml intermittently into the external jugular vein. A mid-line abdominal incision was made and the small intestine was exteriorized. Two loops, each of 25 to 35 cm in length were tied off in the terminal ileum and a polythene cannula was inserted at each end of the loops. The remainder of the small intestine was returned to the peritoneal cavity, ensuring that these prepared loops were not disturbed. The exposed gut was kept moist with warmed saline throughout the experiment. Toxin solution, either 1.5 ml V. cholera 12 r toxin solution (prepared as described in Section 1, Part 1) or Wyeth lot 002 toxin solution (0.2 ml) containing 500 mg toxin per ml, was injected into the test (toxin-challenged) loops and 1.5 ml saline or heat-inactivated Wyeth 002 toxin (0.2 ml of a 500 mg/ml stock solution) was injected into the corresponding control loop. Indomethacin was administered prior to toxin or control solutions as indicated below. The loops were clamped off from inflow and outflow cannulae for 1 hour following toxin administration.

The loops were then perfused for 5 to 6 hours with saline (V. cholerae 12 r toxin loops) or salt solution as prepared by Pierce (Pierce et al, 1971) (Wyeth lot 002 toxin treated loops). The volume secreted or reabsorbed during each hour of the perfusion was measured. The loops were perfused at a rate of 1.5 - 1.9 ml per minute.

Indomethacin Treatment

The seven animals were divided into three groups on the basis of toxin and drug treatment schedules. The dose of indomethacin used was 100 µg per cm of intestinal tissue. Two factors were considered when this dose was selected for pre-treating the intestinal loops. Firstly, the dose had to be such that the amount

of drug absorbed by the epithelial cells was sufficient to reach the microsomal prostaglandin synthetase enzyme and cause appreciable if not total inhibition of the enzyme. The second factor was the potency of indomethacin as a prostaglandin synthetase inhibitor. Flower and his associates (Flower, Gryglewski, Herbaczynska-Cedro & Vane, 1972) demonstrated that the $1D_{50}$ of splenic synthetase in dogs was 0.06 μ g. The assay was performed with purified enzyme and the dose was approximately equivalent to 0.06 μ g per gram wet weight whole spleen. Lung synthetase also exhibits great sensitivity to this drug, being inhibited 50% by a dose of 0.27 μ g indomethacin per ml partially purified enzyme. This was a 0.75 μ M solution of indomethacin, (Vane, 1971). The dose of indomethacin used in these experiments in rabbit perfused loop models of cholera was 3mg per loop which was approximately a dose of 100 μ g per gram wet weight of intestine. It was therefore felt that, even allowing for the possibility that intestinal prostaglandin synthetase might be less sensitive than those in lung and spleen, and that absorption of the drug by the epithelial cell might be poor, marked depression of fluid secretion was likely to be achieved with this dose of indomethacin if prostaglandins were an essential link in the chain of events initiated by cholera toxin which led to fluid secretion in the small intestine.

Group A

3 rabbits were used as controls. The two intestinal loops were not treated with either indomethacin or polyethylene glycol (the vehicle used for drug administration). One loop was inoculated with 1.5 ml V. cholera 12 r/T30/N100 toxin or 0.2 ml Wyeth 002 cholera toxin (0.5g/ml). The control loop was injected with either 1.5 ml saline or 0.2 ml boiled, inactive Wyeth 002 toxin, depending

Rabbit Ileal Loop

Table 9a. The effect of indomethacin on the action of cholera toxin in the perfused ileal loop model of cholera in rabbits.

T = Toxin. Wyeth 002/vc12r

I = Indomethacin. 3mg/ml/loop

C = Control. Boiled Wyeth 002/ 5% Difco Bactopeptone Water
Salt solution from vc12r medium

Note. Values given are the mean + S.E. of 5 experiments for toxin only treatment, 4 for control only, 2 for control + indomethacin and 4 for toxin + indomethacin. "+" denotes volume secreted ; "-" volume reabsorbed.

	Volume (ml)	Volume (ml)	Volume (ml)	Volume (ml)
Perfusion Hours	Toxin (n = 5)	Toxin + Indomethacin (n = 4)	Control (n = 4)	Control + Indomethacin (n = 2)
1	-4.8 ± 0.7	-5.83 ± 0.9	-2.25 ± 1.3	-6.0 ± 2
2	+0.7 ± 1.2	-1.66 ± 1.70	-3.25 ± 0.85	-6.5 ± 3.5
3	+1.9 ± 0.33	+1.0 ± 2.5	-3.75 ± 1.03	-3 ± 1
4	+2.5 ± 0.67	+1.3 ± 1.7	-4.25 ± 0.25	-1.5
5	+2.8 ± 0.37	+2.8 ± 0.17	-2.13 ± 0.83	-0.5
6	+3.5 ± 1.5	(n = 1)+5 ml		

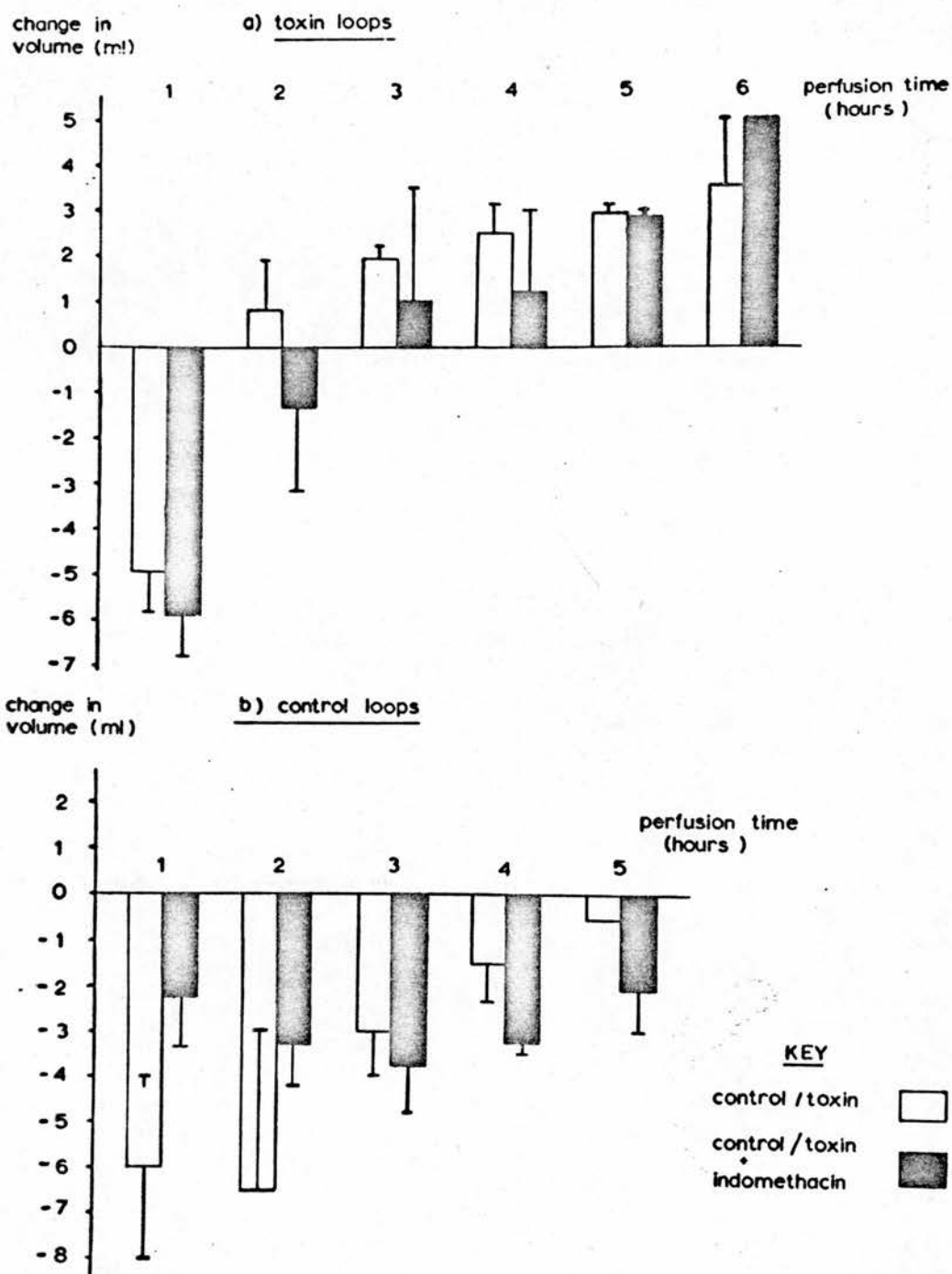


Figure 11. The effect of indomethacin 3 mg/loop on intestinal handling in toxin-treated and control perfused ileal loops in rabbits. Values represent the mean figures \pm S.E.M. For details see Table 9a.

on the volume and nature of the active toxin used in that particular animal.

Group B

As in Group A one of the two loops was used for the injection of cholera toxin while the other served as a control. The two animals in this group received local indomethacin in the toxin loop and in the control loop. The dose of indomethacin used was 3 mg/loop, which was approximately 100 μ g/g wet weight of intestine. The drug was dissolved in 0.1 ml polyethylene glycol. The injection of indomethacin was given 40 minutes prior to toxin challenge.

Group C

There were two animals in this experimental group. Both loops were inoculated with cholera toxin. One was pretreated with indomethacin 3 mg, the other received 0.1 ml polyethylene glycol, the vehicle for indomethacin, 40 minutes before toxin challenge.

Results and Discussion

The results of indomethacin treatment in this series of experiments are summarized in Table 9a and Figure 11.

In all of the loops, both those receiving cholera toxin and those receiving control solution showed a net absorption of fluid during the first hour of the perfusion. The volumes adsorbed were 4.8 ml \pm 0.7 ml (mean of 5 observations \pm SEM), 5.8 \pm 0.9 ml (mean of 4 observations \pm SEM), 2.25 ml \pm 1.3 ml (mean of 4 observations \pm SEM) and 6.0 ml (average of 2 readings) for toxin treated loops, toxin loops pretreated with indomethacin, control and control loops pretreated with indomethacin respectively. Thereafter toxin treated loops showed a tendency to secrete fluid. In the second hour the loops treated with toxin alone secreted 0.7 ml \pm 1.2 ml (mean \pm SEM of 5 results) and toxin loops pretreated

with indomethacin absorbed $1.7 \text{ ml} \pm 1.7 \text{ ml}$ (mean of 4 results \pm SEM).

The difference was not statistically significant ($p > 5\%$)*when

statistical analysis using the Student 't' test was carried out.

During the 3rd, 4th, 5th and 6th hours both sets of toxin loops were observed to secrete fluid. Control loops absorb^{ed} fluid throughout the entire experimental period. In the case of indomethacin pretreated control loops, fluid secretion was observed in 1 loop during the second hour of perfusion. False positive results have been reported in ligated loops (McNaught & Roberts, 1958). The abrupt nature of this response together with the magnitude of the absorption in the first and third hours (-5.5 ml and -6.5 ml) suggest that the secretion of 1.5 ml is likely to be artefactual, rather than a real response to indomethacin treatment.

Conclusion

Local administration of indomethacin, $100 \mu\text{g/g}$ wet weight intestine, to the lumen of the intestine forty minutes before toxin challenge, did not inhibit the secretory response of the intestine. At this dose the ability of the intestine ^{to reabsorb fluid} did not appear to be compromised during the 1st and the 3rd to 6th hours of perfusion. During the second hour the intestine was observed to secrete fluid in one rabbit. This was attributed to artefact rather than a real response.

* The actual value for p was 7% ;when the Bessel correction was applied (to take account of the small numbers involved) the value of p was increased to 31%.

Experiment 5

The in vitro synthesis of prostaglandins by rabbit intestine and the effect of cholera toxin on this synthesis

It had been observed that in the ligated loop model of experimental cholera, that prostaglandin release did occur following cholera toxin challenge. No such release was obtained in the perfused loop model. Indomethacin did not inhibit the secretory activity in either of these models, which strongly suggested that prostaglandins did not have an obligatory role in the fluid secretion stimulated by cholera toxin. It was considered possible, though not very likely, that cholera toxin might stimulate prostaglandin synthetase activity which was resistant in some way to the effects of indomethacin. In this experiment and in the following experiment it was intended to investigate this possibility. In this experiment the effect of cholera toxin on the in vitro synthesis of PGE-like and PGF-like compounds was studied. In the subsequent experiment the effect of indomethacin on the biosynthesis was observed.

Methods

Terminal ileum was removed from 12 rabbits at death. The tissue was handled as little as possible and kept on ice whenever this was feasible in the period prior to incubation. After freeing the intestine from mesentery, fat and blood vessels, the section of gut was chopped into small pieces and weighed. It was washed and then transferred to conical flasks containing ice-cold Tyrode solution. The volume of Tyrode used in all experiments was 10 ml per gram wet weight of tissue. The preparation was gassed with oxygen and incubated at 37°C for three hours.

Purified cholera toxin solution containing 75 mg-150 mg

225B cholera toxin was added to the flasks to give a final concentration of 75 μ g-150 μ g per 100 ml Tyrode's solution. Six flasks were incubated with active toxin. The control flasks were inoculated with an equal volume of 0.9% saline, the solution in which the toxin had been dissolved.

The reaction was terminated by plunging the flasks and contents into an ice-bath and acidifying the incubate to pH 3.5-4.0 with 10% acetic acid. The supernatant was harvested and subjected to prostaglandin extraction procedures as described in earlier experiments.

Extraction, purification and separation of prostaglandins

The extraction of samples was achieved using method B described in Experiment 1. The dried residue obtained after the 67% ethanol extract was evaporated to dryness under reduced pressure was stored at -15°C until column chromatography was performed. It was necessary to modify the solvent systems used to elute the silicic acid columns because freshly sieved batches of Sigma SIL R, 100-200 mesh and Bio-Rad SIL A, 100-200 mesh batch 6910 silicic acid were used. The solvent systems employed to achieve discrete separation of PGE and PGF compounds on chromatography with the new batches of acids are shown in Table 10a and Table 10b. Standard prostaglandins were dissolved in 30% ethyl acetate in toluene and applied to the top of the columns as described in the Methods Section of Experiment 1. The columns were eluted at a rate of 10-15 drops in 20 seconds which corresponded to a flow rate of 1 ml per minute. The fractions were collected separately and evaporated to dryness. They were redissolved in 1 ml distilled water and assayed on the rat fundal strip. Using the solvent systems described, PGE was eluted in the 65% ethyl acetate fraction of 130 ml

and 160 ml volumes and PGF was present in the 80% ethyl acetate fraction only.

Biological Assay

Stock solutions of standard PGE_1 and $\text{PGF}_{2\alpha}$ containing 100ng ml of the respective prostaglandin were prepared. The rat fundal strip was used to estimate the PGE and PGF content of the desiccated column eluates. The fractions were dissolved in 1.0 ml distilled water and assayed against standard PGE_1 and $\text{PGF}_{2\alpha}$ in the case of the PGE and PGF fractions respectively. Contractions of the fundus to standard PGE_1 and $\text{PGF}_{2\alpha}$ were obtained and the responses of the tissue to aliquots of the samples was compared to these. A three minute dose cycle was employed for the PGE assay and the drug contact time was 45-60 seconds. When $\text{PGF}_{2\alpha}$ was the stimulant of contraction it was necessary to lengthen the dose cycle to $3\frac{1}{2}$ -4 minutes and to allow 60 - 90 seconds drug contact time. The method of estimation was the same as in previous experiments.

Statistical Analysis

The statistical significance of the results was assessed by student 't' test.

Results

The prostaglandin synthesized in the presence and absence of cholera toxin by rabbit terminal ileum, in vitro, was estimated in terms of PGE_1 and $\text{PGF}_{2\alpha}$ equivalents. This does not exclude the synthesis of other prostaglandins in the E and F series. The mean PGE synthesis in toxin-treated tissue was 42.2 ± 8.1 ng/g wet weight tissue for three hours. Control tissue synthesis was 46.5 ± 17.3 ng/g PGE_1 equivalents wet weight tissue per three hour incubation period. The difference was not statistically significant. Synthesis of prostaglandin F-like

Table 10 (a). The solvent system developed for use with Sigma-
SIL-R 100-300 mesh sieved silicic acid

Fraction Number	Volume (ml)	Percentage Ethyl Acetate	Percentage Toluene	Percentage Methanol
1	40	30	70	-
2	40	40	60	-
3	20	65	35	-
4	160	65	35	-
5	20	65	35	-
6	160	80	20	-
7	50	100	-	-
8	50	-	-	100

Table 10 (b). The solvent system developed for use with Bio Rad -
SIL A 100-200 mesh batch 6910, sieved silicic acid

Fraction Number	Volume (ml)	Percentage Ethyl Acetate	Percentage Toluene	Percentage Methanol
1	40	19.9	80	0.1
2	40	29.8	60	0.2
3	130	64.3	35	0.7
4	20	64.3	35	0.7
5	130	79	20	1.0
6	40	99	-	1.0
7	40	-	-	100

material also showed no significant alteration when cholera toxin was added to the incubate, during the three hour incubation ($p > 5\%$). In the presence of the toxin the $\text{PGF}_{2\alpha}$ -like activity detected in the supernatant was 21.3 ± 0.7 ng per wet weight of tissue whilst the corresponding figure for tissue containing no cholera toxin was 23.3 ± 3.1 ng/g wet weight tissue/three hour incubation period. The results of this experiment are summarized in Table 11 and Figure 12.

Conclusion

Purified cholera toxin prepared from *Vibrio cholera* 225B did not appear to influence the in vitro synthesis of PGE_1 -like and $\text{PGF}_{2\alpha}$ -like compounds by rabbit terminal ileum.

Table 11. The synthesis of prostaglandins in vitro

Experiment	Control PGE ₁ ng/g/3 hr ¹	Control PGF _{2α} ng/g/3 hr ^{2α}	Toxin PGE ₁ ng/g/3 hr ¹	Toxin PGF _{2α} ng/g/3 hr
1	71.0	23.66	57.1	21.4
2	64.0	29.07	39.4	21.1
3	30.0	20.0	32.7	20.0
4	38.8	22.2	39.2	21.6
5	30.5	21.4	41.6	22.2
6	44.5	23.2	42.9	21.4
Mean	46.46	23.33	42.15	21.28
S.E.M.	7.09	1.27	3.32	0.29
S.D.	17.3	3.10	8.12	0.72
"p" tox-control	N.S. p > 5%	N.S. p > 5%		

Rate of synthesis
ng/g/3hr.

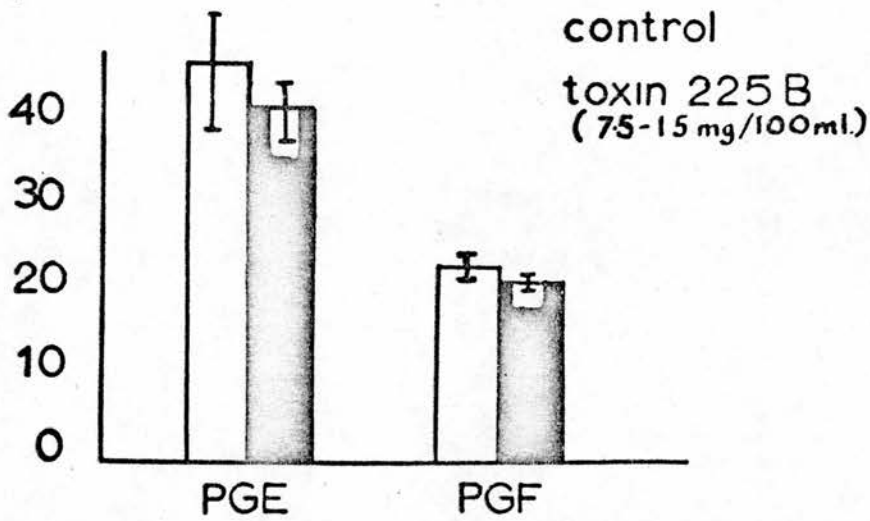


Figure.12 The in vitro synthesis of prostaglandins and the effect of purified cholera toxin. Mean values \pm S.E.M. of 6 experiments.

Experiment 6

The inhibition of in vitro prostaglandin biosynthesis by indomethacin in cholera toxin-challenged and control segments of rabbit terminal ileum

The doses of indomethacin used in the in vivo experiments were 10-40 mg/kg body weight, in the case of systemic administration of the drug. Local administration doses were approximately 100 µg/g wet weight intestine. The peak plasma levels achieved with systemic indomethacin were estimated to be of the order of 8 to 30 µg/ml plasma, assuming total body water distribution of the drug. In two experiments the effect of incubating tissue with 10 µg/ml and 15 µg/ml indomethacin was investigated in toxin/-treated chopped intestine. The effect of 15 µg/ml indomethacin on prostaglandin production by control tissue was also studied in one experiment.

The procedure was exactly the same as that adopted in the previous experiment with the exception that indomethacin (10 µg or 15 µg per ml of incubate) was added prior to incubation and before toxin or control medium was added to the flasks.

Extraction, Purification and Separation of Prostaglandins

Prostaglandin E and F containing fractions were obtained by the methods outlined in Experiment 5.

Biological Assay

Samples were assayed on the rat fundal strip as described in Experiment 5 in terms of standard PGE₁ and PGF_{2α}.

Results

Indomethacin produced marked inhibition of prostaglandin biosynthesis when added in doses of 10 µg and 15 µg per ml to the

intestinal incubate. Both toxin treated and control tissue synthesis was affected. The lower dose of indomethacin inhibited PGE synthesis by 66.8% and PGF synthesis by 82.5% as estimated in terms of standard PGE₁ and PGF_{2α}. Indomethacin 15 µg/ml caused a further reduction in prostaglandin production. The percentage inhibition was 77.5% and 83.7% for PGE₁ and PGF_{2α} respectively in the toxin challenged preparations. Control intestine biosynthesis was reduced by 76.7% in the case of PGE₁ and by more than 65% for PGF_{2α} with a dose of 15 µg/ml indomethacin. There was no detectable PGF_{2α} -like activity contained in the sample. The limit of the assay was 7ng PGF_{2α} per gram of tissue. The results are contained in Table 12.

Table 12. The effect of indomethacin on the in vitro synthesis of prostaglandins in the toxin-treated and non toxin-treated chopped intestine of rabbits

Sample	PGE ₁ produced ng/g	PGF _{2α} produced ng/g	% Reduction	
			PGE ₁	PGF _{2α}
Toxin	39.4	21.1	66.8%	82.5%
Toxin + Indom. 10µg/ml	13.1	3.7		
Toxin	57.1	21.4	77.5%	83.7%
Toxin + Indom. 15µg/ml	12.9	3.5		
Control	30.0	20.0	76.7%	65%
Control + Indom. 15µg/ml	7.0	7.0		

Conclusion

Prostaglandin synthetase in toxin-treated tissue is not resistant to the effect of the prostaglandin synthetase inhibitor, indomethacin. Indomethacin in concentrations liable to be present in rabbit plasma following the intravenous injection of doses of 10-40 mg/kg in vivo is capable of causing significant inhibition of prostaglandin production by the small intestine in vitro.

Experiment 7. The Selection of Doses of Wyeth 002 Toxin for use in the luminally perfused Ileal Loop Model of Cholera in Cats

The rabbit models had to be abandoned for reasons beyond the writer's control. It was therefore decided to pursue the investigations on the role of prostaglandins in cholera using another experimental animal. The cat was selected because it had been observed to be sensitive to the effects of cholera toxin and secreted water and electrolytes in response to intraluminal injection of the toxin in the small intestine.

Before embarking on a long series of experiments using this model it was essential to select a dose of toxin that would be capable of stimulating fluid secretion. The toxin which was to be used in these animals was the Wyeth crude cholera toxin Wyeth lot 002 that had been used in some of the rabbit experiments previously described (Experiments 3 and 4, Section 1, Part 2). The ligated intestinal loop model is a useful preparation in which to study the sensitivity of the small intestine to the toxin. It has been used in rabbits where a dose dependency of the response has been observed following challenge with other toxin preparations. (for references see Introduction).

Methods

One young male cat weighing 2.0 kg was anaesthetized with pentobarbitone sodium 40 mg/kg. During the course of the experiment anaesthesia was maintained by supplementary intravenous injections of the anaesthetic. Tracheostomy was performed and the blood pressure was monitored using a Grass Polygraph connected to a Stantham pressure transducer which registered carotid arterial blood pressure.

A midline abdominal incision was made and the small intestine was carefully exteriorized. Four ~~ligated~~ loops were tied off using

mersilk ligatures in the terminal small intestine. Each was 10-12 cm in length and was separated from the next by a gap of 2-3 cm in length. The loops were injected intraluminally with the toxin in doses of 50 mg, 100 mg, 500 mg and 1g respectively. The volume used for the injections was the same for all loops and was 2 ml. The toxin was dissolved in 0.9% saline. The intestine was restored to the abdominal cavity and the incision was lightly sutured. The loops were examined three hours later and the presence of fluid in the lumen was noted.

Results

No fluid accumulation was observed in the loops challenged with the 50 mg and 100 mg doses of toxin. The section of intestine which had been injected with the 500 mg dose of Wyeth toxin did contain fluid, as did the loop challenged with 1 g crude toxin material. The volumes secreted were 2.8 ml and 3.5 ml respectively.

Discussion

The 50 mg and 100 mg doses of toxin were ineffective and did not appear to cause any change in gastro-intestinal function as far as the handling of water and electrolytes was concerned. Cholera toxin is known to be a potent stimulator of fluid secretion in the small intestine (see Introduction). The toxin used in this series of experiments had been stored for some considerable time (6 months) and was therefore likely to have lost some of its activity. That a complete loss of enterotoxic activity had not occurred was evident from the secretion of fluid in the loops challenged with the higher doses of toxin, namely 500 mg and 1 g toxin. A longer interval between toxin administration and observation

of the secretory response of the small intestine to the toxin might have resulted in a positive response occurring in loops receiving the lower doses of toxin. The purpose of the experiment was not however to determine the absolute level of sensitivity of the cat intestine to cholera toxin but to select a dose of toxin which would give a clearcut secretory response in a loop of terminal ileum within a 5-6 hour perfusion period. For this to occur it is necessary that the onset of secretion should occur in the first or second hour of exposure to the toxin. The doses of 500 mg and 1 g of toxin fulfilled these conditions and were therefore suitable for use in this series of experiments.

Conclusion

The cat small intestine shows a dose-dependent secretory response to cholera toxin challenge. Low doses of crude toxin (50 and 100 mg) are not capable of stimulating fluid secretion in the small intestine of cats within three hours. Higher doses of toxin (500 mg and 1 g) elicit a secretory response from intestinal tissue in this time interval and are therefore suitable doses for investigating the effect of cholera toxin on prostaglandin release and fluid production in the cat perfused loop model of cholera.

Experiment 8. The stimulation of fluid secretion in the cat small intestine by cholera toxin

In the previous experiment doses of 500 mg and 1 g Wyeth crude, 002 toxin was found to stimulate fluid production in the terminal small intestine in cats. The ligated loop was not considered to be the most useful model for investigating the role of prostaglandin release in fluid secretion during cholera due to the effects of loop distension and haemorrhagic necrosis on prostaglandin release by the intestinal mucosa. A perfused loop model similar to that used in the rabbit (Experiments 3 and 4) was therefore used and a dose of 1g cholera toxin was selected.

Methods

Four young cats and kittens were used. Anaesthesia was induced with pentobarbitone sodium, 40mg/kg, intravenously administered, and was maintained by a continuous intravenous infusion of pentobarbitone sodium (6mg/ml) at rates varying between 0.075-6.6ml/hr. The anaesthetic solution contained heparin 100iu/ml. The right common carotid ^{artery} was cannulated for blood pressure recording on a Grass polygraph. Tracheostomy was performed and the animals were artificially ventilated if respiration became unsatisfactory. The right femoral vein or external jugular vein was used to inject anti-inflammatory drugs in later experiments and was therefore cannulated in this series of animals although it was not used.

A mid-line abdominal incision was made and the terminal small intestine was exteriorized. Three loops were made using Mersilk ligatures in the small intestine. The last of these extended to a point 15-20 cm proximal to the ileo-caecal junction. Both the first and the third loops were approximately 30 cm in length while

the second was 5-8 cm long. The longer loops were cannulated at both ends and challenged with toxin or control solution. The intervening loop was not operated on as it was merely intended to separate the perfused segments of gut.

One gram of crude Wyeth 002 cholera toxin was dissolved in salt solution (10 ml) and injected into the test loop. Control loops were challenged with an equal volume of salt solution. The salt solution used in this series of experiments was prepared according to Pierce and his associates (Pierce et al, 1971). The loops were clamped off from the inflow reservoir for one hour. The clamps were removed and the loops were perfused at a rate of 1.0-1.7 ml/minute for five hours. The fluid was collected at the end of each hour of perfusion and the net amount either secreted or reabsorbed was determined. The fluid was stored at -15°C until a prostaglandin extraction could be carried out after each perfusion period and fresh salt solution was circulated through the loops. In each pair of experiments the test and control loops were reversed in position relative to the ileo-caecal junction in a cross-over design thus ensuring an equal number of test and control values for a given region of gut.

Results

The effect of cholera toxin on the small intestine was investigated in 4 cats. The results are summarized in Table 13 and Figure 13. The toxin-challenged loops exhibited a secretory response to the toxin throughout the 6 hours of perfusion. The average output varied from 1.3 ml per hour to 7.1 ml per hour. The mean output was 4.5 ml per hour over the perfusion period. Control loops, by contrast, did not secrete fluid but absorbed

Table 13. The effect of cholera toxin on gastro-intestinal tract handling of salt solution perfused through the lumen of loops in the lower small intestine in cats

Toxin Treated Loop			Control Loop		
Hours of Perfusion (hours)	Volume secreted mean \pm S.D. (ml)	S.E.M.	Hours of Perfusion (hours)	Volume absorbed mean \pm S.D. (ml)	S.E.M.
1	6.8 \pm 1.0 (n = 4)	0.5	1	- 13.5 \pm 13.6 (n = 4)	6.8
2	7.1 \pm 3.6 (n = 4)	1.8	2	- 5.9 \pm 4.2 (n = 4)	2.1
3	5.0 \pm 2.4 (n = 4)	1.2	3	- 3.0 \pm 2.5 (n = 4)	1.2
4	3.8 \pm 4.3 (n = 4)	2.1	4	- 5.5 \pm 4.6 (n = 4)	2.3
5	1.3 (av. of 2)		5	- 7.5 \pm 7.0 (n = 3)	4.1
6	3 (one observation)		6	- 6.5 (one observation)	

Average
change in
volume per
hour

4.5 ml

- 7 ml

Control Experiment. The effect of cholera toxin on intestinal handling of perfusate.

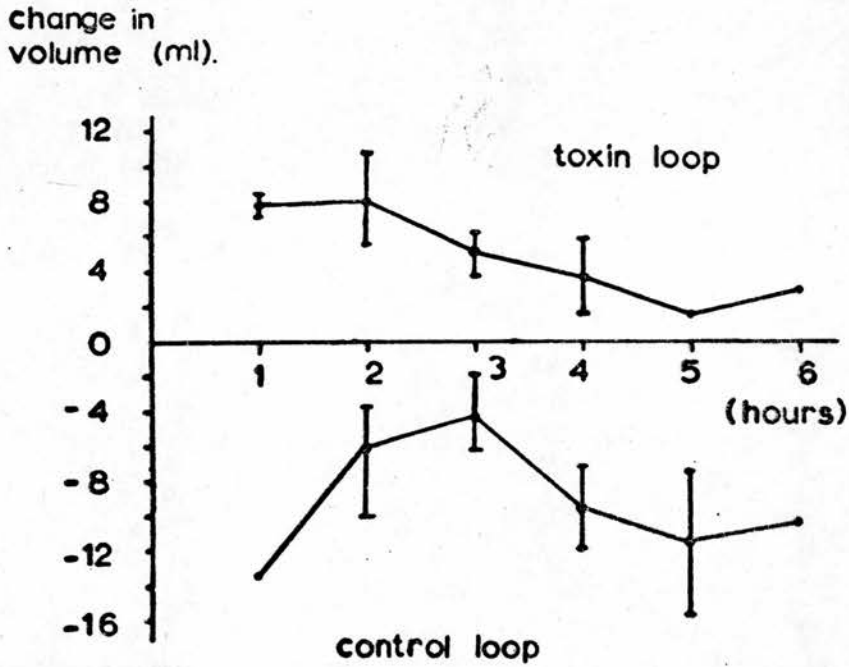


FIGURE 13.

Points represent mean values (see Table 13) \pm S.E.M.

water and electrolytes from the solution perfusing the lumen of the loop. Reabsorption of 3 to 13.5 ml per hour occurred. The average reabsorption per hour was 7 ml per hour.

There was considerable variation between different animals in the extent of fluid secretion and reabsorption but the general trend of the response was always the same: toxin challenge causing fluid loss; lack of toxin resulting in fluid absorption.

Conclusion

These experiments clearly established that cholera toxin stimulates fluid secretion in terminal small intestine loops in cats. The effect is localized to the loop receiving toxin as is seen from the continued reabsorption of perfusate in the unchallenged loop. The effect seen is similar to that observed in rabbits using purified cholera toxin .225B and crude V. cholerae 12r/T30/N100 toxin (Experiments 3 and 4).

Experiment 9

The effect of prophylactic administration of indomethacin and aspirin on fluid production in response to cholera toxin

In rabbits indomethacin pretreatment failed to abolish the secretory response of the intestine to cholera toxin. It was therefore of interest to see if cats, which also respond to cholera toxin intestinal challenge by secreting water ^{and} electrolytes, were sensitive to systemic and local drug application and showed a decreased fluid output following drug administration. The effects of aspirin (25 mg/kg) were also investigated. Aspirin, like indomethacin, is an inhibitor of prostaglandin biosynthesis (Vane, 1971).

Methods

Seventeen cats weighing from 1.3 to 3.75 kg were pretreated with either indomethacin or aspirin. The experimental procedure was exactly the same as in experiments where no drug treatment was given. The drugs were administered either by intravenous injection (for indomethacin and aspirin) or by local injection into the lumen of the small intestine (indomethacin).

Indomethacin was dissolved in 0.2 ml polyethylene glycol and the volume was made up to 2.5 ml with saline (0.9%) for intravenous injection. When local administration of indomethacin was carried out, the drug was dissolved in polyethylene glycol only. The doses of indomethacin employed in these experiments were: 10mg/kg, 50mg/kg and 100mg/kg given by the intravenous route to 5, 3 and 1 animal respectively. Three animals received local injections of 600 µg per loop of the drug into the lumen of the small intestine. Only one dose of aspirin was used - 25 mg/kg and this was given as a single intravenous injection, as was

indomethacin, except in 2 cats when the initial 10mg/kg dose was supplemented by 2 further 10mg/kg doses at 2 and 4 hours after toxin challenge.

The drugs which were administered systemically were injected slowly over a two minute period ten minutes before the toxin was administered. Local indomethacin was delivered to the loop twenty minutes before toxin or control solution was introduced into the loop.

Fluid was circulated through the toxin and control loops as described in the previous experiment and the effect of the drugs on the intestinal handling of fluid following toxin challenge was noted and compared to that of the control segments of gut.

Perfusate was collected at the end of each hour of perfusion and stored at -15°C until such time as extraction could be carried out.

Results and Discussion

The volume secreted or absorbed during each hour of perfusion, following the one hour initial 'incubation' period when the loop was clamped off from the inflow and outflow cannulae, by the intestinal loops was measured. The mean volume for each group of animals during each hour was calculated. Individual animals showed a considerable variation in their sensitivity to cholera toxin, but it was evident that toxin-challenged loops showed a marked difference in behaviour to the control, unchallenged loops. The former showed secretory activity whereas the latter absorbed water and electrolytes from the solution which was perfused through the intestinal lumen. The results are summarized in figures 14 to 17 and Tables 14 to 17.

(a) Animals treated with intravenous indomethacin

I. Animals receiving Indomethacin 10mg/kg

Five animals were treated with indomethacin in a dose of 10mg/kg. Three animals received a single intravenous dose prior to toxin challenge whilst the other two were given a supplementary 10mg/kg dose two hours after toxin challenge. As these latter cats did not exhibit a different pattern of response to animals given the single prophylactic dose, the results were treated together. The mean fluid output per hour in this group of animals was 4.9ml/hour in the toxin loop. The control, non-toxin loop exhibiting an entirely different pattern of behaviour during the perfusion period; this loop reabsorbed fluid. The mean hourly absorption was 7.6 ml per hour. Neither of these values differs significantly ($p > 5\%$) from the pattern of fluid movement observed in the corresponding loops of animals which were not subjected to either indomethacin or aspirin treatment (Experiment 8; Results Section). These animals showed a mean secretion of 4.5 ml in toxin challenged loops and a mean absorption of 7.0 ml in the control loops.

(2) Animals receiving 50 mg/kg Indomethacin

A single intravenous injection of 50 mg/kg bodyweight indomethacin failed to suppress the secretory stimulus exerted by cholera toxin on the terminal ileum. Toxin treated loops showed a mean secretion of 4.4 ml fluid per hour, whereas the control loops were found to absorb fluid to the extent of 5.8 ml per hour. The absorption figure is lower than the mean value for reabsorption in other control loops. The control loops in the animals receiving this high dose (50 mg) indomethacin, became engorged with blood and damaged so that observations could not be extended beyond the first

four hours of perfusion. The low mean absorption figure thus reflects in part this trauma. The individual loops did show a wide variation in absorption capacity but up to four hours did not differ significantly in their ability to absorb fluid as compared to other control, non-toxin treated loops in other animals. The mean absorption figure was not significantly different from the mean absorption figure of non-drug, non-toxin treated intestinal loops ($p > 5\%$).

(3) Animals receiving 100 mg/kg Indomethacin

One animal received this high dose of indomethacin. The animal's blood pressure fell markedly following this dose and remained depressed throughout the experimental period. Nevertheless, no inhibitory effect on fluid production was observed. The toxin challenged loop showed a net fluid output of 11, 3, 3, 2 and 6 ml in the five hours of perfusion. The control loop reabsorbed fluid. Only 3 measurements could be made owing to the loop becoming fragile and haemorrhaging. These three hour absorption volumes were 8, 5 and 5 ml respectively. The mean secretory volume per hour was 5.0 ml per hour and the absorption amounted to 6 ml per hour. Neither of these is significantly different to the results obtained for the corresponding loops in Experiment 8 where no drug treatment was employed ($p > 5\%$).

(b) Animals treated with locally applied indomethacin 600 ug/loop

In this group of animals both loops were exposed to cholera toxin but one loop received a local intraluminal injection of indomethacin 600 μ g/loop twenty minutes prior to toxin challenge. The dose was selected on the grounds that it was 4 orders of magnitude greater than that found to give 50% inhibition of prostaglandin synthesis in dog spleen by Flower and his associates (Flower et al, 1971). It

Table 14. The response of cholera toxin-challenged loops and Control intestinal loops in cats to indomethacin pretreatment (10 mg/kg by intravenous route)

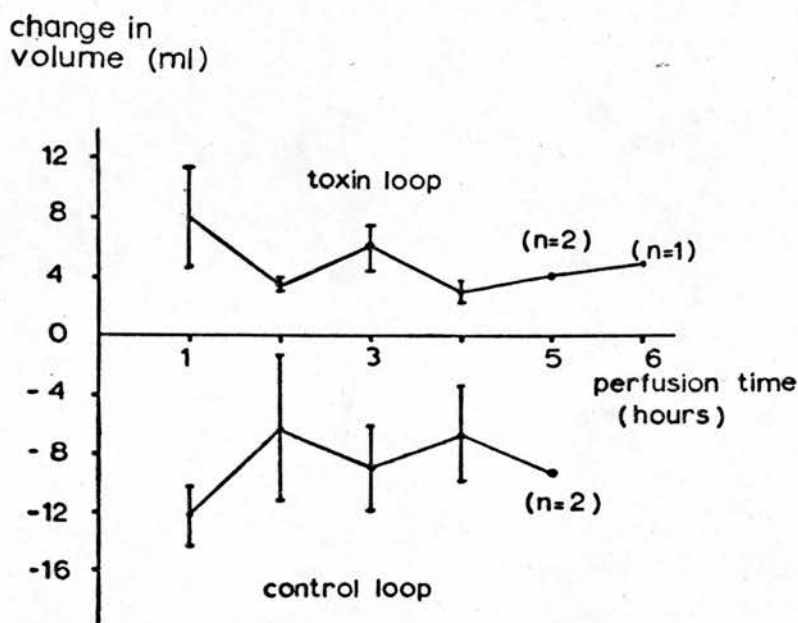
Indomethacin 10mg/kg. I.V. Injection				
Toxin-challenged loop			Control Loop -ve sign indicates reabsorption	
Hour of Perfusion (hour)	Change in Volume mean \pm S.D. (ml)	S.E.M. (ml)	Change in Volume mean \pm S.D. (ml)	S.E.M. (ml)
1	8 \pm 6.7	3.4	-12.2 \pm 4.0	2.3
2	3.3 \pm 0.6	0.3	-6.3 \pm 9.2	5.3
3	6.0 \pm 3.4	1.7	-9.1 \pm 5.5	2.8
4	3.0 \pm 1.0	0.6	-6.7 \pm 5.5	3.2
5	4.0 (2 observations)		-9.5 (2 observations)	
6	5.0 (1 observation)		-	
Mean change	4.9 ml/hr		-7.6 ml/hr	

Table 15. The response of cholera toxin-challenged and control loops in the terminal ileum in cats following systemic pretreatment with indomethacin 50mg/kg

Toxin-challenged loop			Control Loop	
Hour of Perfusion (hours)	Change in Volume mean \pm S.D. (ml)	S.E.M.	Change in Volume mean \pm S.D. (ml)	S.E.M.
1	8.7 \pm 3.8	2.2	-8.0 \pm 1.0	0.6
2	5.7 \pm 1.2	0.7	-7.3 \pm 4.9	2.8
3	2.7 \pm 2.1	1.2	-3.0 \pm 5.0	2.9
4	3.0 \pm 1.0	0.6	-5.0 \pm 4.0	2.3
5	2 (1 observation)		-	
6	4 (1 observation)		-	
Mean change	4.4 ml/hr		-5.8 ml/hr	

FIGURE 14.

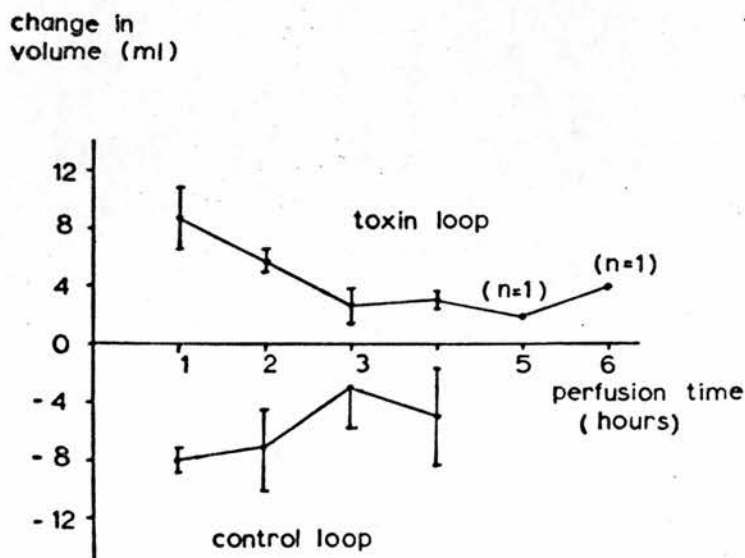
The effect of indomethacin 10mg kg i.v.
on intestinal handling of perfusate.



Points represent mean values (see table 14) \pm S.E.M.

FIGURE 15.

The effect of indomethacin 50mg kg i.v.
on intestinal handling of perfusate.



Points represent mean values (see table 15) \pm S.E.M.

was therefore likely to inhibit the biosynthesis of prostaglandins even if absorption was poor and the intestinal prostaglandin synthetase was particularly insensitive to this inhibitory agent. There was however no depression in fluid output by either loop. The average output per hour was 5ml in the case of the loops treated with toxin alone and 4.2ml in the toxin loops pretreated with indomethacin. Neither of these figures is significantly different from the values obtained in other experiments in which indomethacin was employed, nor are they significantly lower or higher than those in toxin-treated loops of animals receiving no inflammatory agent.

Although fluid output appeared to be depressed during the second hour of perfusion, in the indomethacin-treated loops, this difference was not statistically significant ($p = 11\%$).

All statistical analysis and evaluation of the significance of these results was done by means of the Student's 't' test with the Bessel correction applied to allow for the small numbers in each sample.

It was noted that in all the experiments in which indomethacin was used to modify the secretory response of the small intestine to cholera toxin there appeared to be a decrease in the fluid secreted during the second hour of intestinal perfusion. The depression in the volume output was not statistically significant. The values for p were 7% and 61% respectively in animals pretreated with intravenous indomethacin in doses of 10 mg/kg body weight and 50 mg/kg body weight. A much larger sample would have to be examined in order to determine whether this apparent trend were of any real significance. That the higher dose of indomethacin had less of a depressant effect on fluid output than did the lower one would however seem to argue against this.

(c) Animals treated with intravenous aspirin (25mg/kg).

The mean output of fluid in the aspirin-treated group of 4 cats was 5.8ml/hour. The volume absorbed in the control loops was 6.8ml/hour. From these results it is clear that aspirin in the dose used did not interfere with fluid absorption nor did it inhibit the effect of cholera toxin. The results are summarized in Figure 17 and in Table 17.

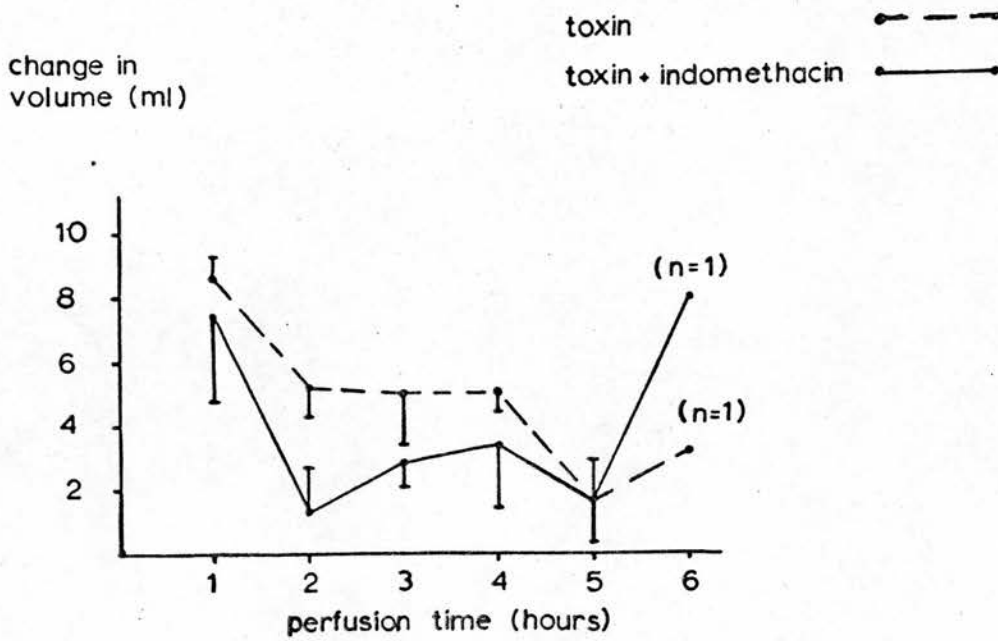
Table 16. The effect of local indomethacin (600 μ g) on fluid output in cat intestinal loops stimulated by cholera toxin. Except where otherwise indicated the results are the mean of 3 observations

Hours Perfusion (Hours)	Toxin Loop		Toxin & Indomethacin	
	Change in Volume Output ml \pm S.D.	S.E.M.	Change in Vol. output ml \pm S.D.	S.E.M.
1	8.5 \pm 1.5	0.9	7.3 \pm 4.6	2.7
2	5.7 \pm 2.1	1.2	1.1 \pm 2.5	1.4
3	5.0 \pm 2.6	1.5	2.8 \pm 1.0	0.6
4	5.0 \pm 1.0	0.6	3.4 \pm 3.7	2.1
5	1.6 \pm 2.1	1.2	1.5 (mean of 2 observations)	
6	4.4 (1 observation)		9 (1 observation)	
Mean/hr	5.0 ml/hr		4.2 ml/hr	

Table 17. The effect of aspirin (25 mg/kg) injected intravenously in the cat terminal ileum response to challenge with cholera toxin

Hours of Perfusion (Hours)	Toxin Treated Loop (n = 4)		Control Loop (n = 4)	
	Volume output mean \pm S.D. (ml)	S.E.M.	Volume output mean \pm S.D. (ml)	S.E.M.
1	11.3 \pm 3.8 (n = 3)	2.1	-12.3 \pm 4.7	2.4
2	6.8 \pm 1.7	0.8	-6.0 \pm 8.6	4.3
3	3.0 \pm 3.2	1.6	-5.0 \pm 2.2	1.1
4	3.5 \pm 1.7	0.9	-3.7 \pm 2.1 (n = 3)	1.2
5	4.5 \pm 1.9	1.0	-7.0 \pm 6.2 (n = 3)	3.6
Mean output	5.8 ml/hr		-6.8 ml/hr	

* (n = 4). The number of observations for each hour of perfusion was 4 except where otherwise indicated.



The effect of indomethacin 600 μ g/loop on intestinal handling of perfusate.

FIGURE 16.

Points represent mean values (see table 16) \pm S.E.M.

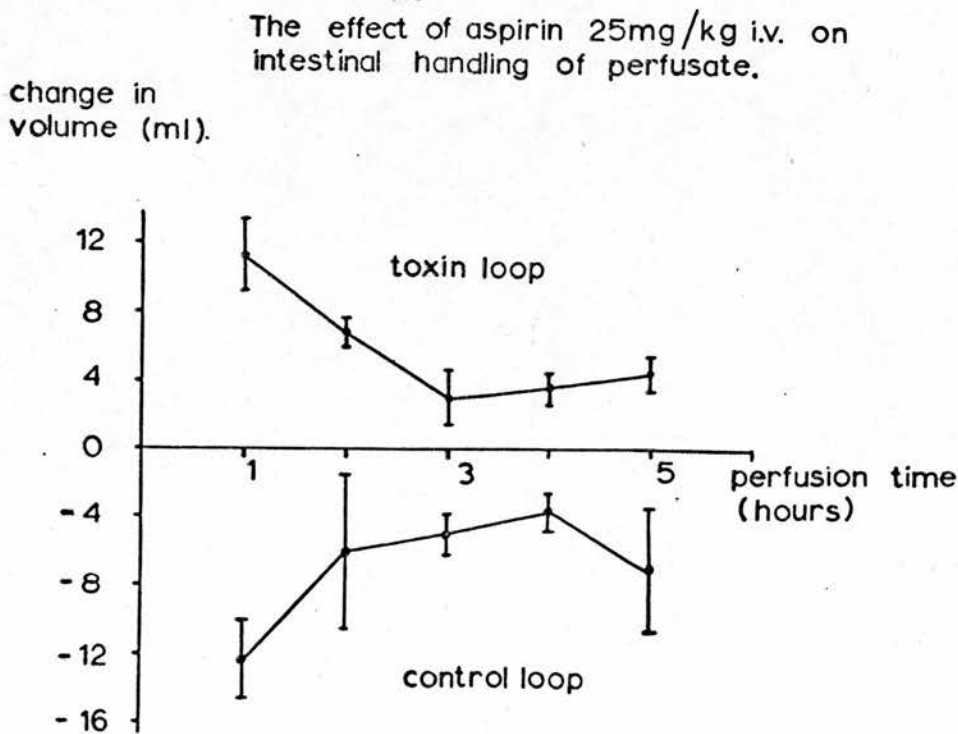


FIGURE 17.

Points represent mean values (see table 17) \pm S.E.M.

Conclusion

Neither indomethacin in doses of 10, 50 or 100 mg/kg given intravenously nor 600 µg indomethacin injected directly into the lumen of the small intestine loop were capable of inhibiting the effect of cholera toxin on the gut. Aspirin in the dose used (25 mg/kg) failed to antagonize the secretory response to cholera toxin. It would thus appear that as in the rabbit, prostaglandin synthesis inhibitors are not able to suppress the choleraic syndrome in experimental animals. Since prostaglandin biosynthesis would be considerably reduced by these drugs, this gives further support to the hypothesis that prostaglandins are not essential factors in the secretion of water and electrolytes in experimental cholera.

Comment on published paper. see Appendix.

Note that the data presented graphically in the published paper are expressed in terms of percentage changes in the volume of fluid output whereas those data presented in this thesis are expressed in terms of absolute volumes of fluid in relation to the perfusion time. The latter form of presentation is preferable on the grounds that it is more likely to avoid errors associated with variations in the perfusion volumes.

Experiment 10

The secretion of fluid in the upper small intestine of the cat and the effect of indomethacin pretreatment on the fluid output

Since this work on cat perfused loop was undertaken, it has been reported by Fink and Katz (Fink and Katz, 1972) that aspirin inhibits fluid secretion in the upper small intestine stimulated by cholera toxin. It was therefore decided to see if the difference between this report and the work outlined in Experiments 9 and 10 was attributable to a regional variation in the sensitivity of the small intestine to cholera toxin and anti-inflammatory drugs. The effect of indomethacin 10 mg/kg given by intravenous injection was investigated.

Methods

Two kittens were used in this series of experiments. One, weighing 1.2 kg, was used as a control animal and received no drug treatment; the other, which weighed 2 kg, was pretreated with indomethacin administered as a single intravenous injection of 10 mg/kg 10 minutes before toxin challenge. The experimental procedure was exactly the same as in the previous cat loop perfusion experiments except that the intestinal loops were made in the upper small intestine. The first of the two loops was made just below the point of entry of the pancreatic duct. The loops were 32 cm long and 35 cm long in the case of the toxin-challenged loops in the untreated animals and drug-treated animals respectively. The control loops were 37 cm long in the animal given indomethacin intravenously. The intestinal lumen was perfused for 4 hours in the animal receiving no drug treatment but for 3 hours only in the indomethacin-treated cat, after the initial 1 hour incubation period of the toxin in the intestinal loop. The dose of cholera toxin used was 1 g in

10 ml salt solution, as in previous experiments, and the control loops were injected with equal volumes of control salt solution (Pierce et al, 1971).

Results

(a) Upper small intestine loop perfusion. No drug treatment.

The cat which received no drug treatment was observed to secrete fluid in response to cholera toxin challenge. The mean hourly output was 3.75 ml per 32 cm length of intestine, which corresponded to a rate of fluid production of 0.1 ml/cm/hour. The control loop in this animal reabsorbed fluid. The extent to which the perfusing solution was absorbed was 8.8 ml per hour (mean of 4 hour measurements). This corresponded to a rate of absorption of 0.2 ml/cm intestine/hour.

(b) Upper small intestine loop perfusion. Indomethacin pretreatment.

Fluid secretion was also observed by the toxin-challenged loop of the animal given indomethacin 10 mg/kg by intravenous injection 10 minutes prior to toxin administration. The mean fluid output in the 3 hour observation period was 4.0 ml/hour for the 35 cm long loop. The rate of fluid production averaged 0.1 ml/cm gut/hour. The control loop reabsorbed fluid during this experimental period. The mean volume reabsorbed per hour was 10.3 ml ~~per hour~~ which corresponded to a rate of 0.3 ml/cm gut/hour.

Conclusion

The rate of fluid production in the indomethacin-treated cat and the untreated animal in response to cholera toxin were almost identical. The control loop in the indomethacin-treated animal showed a slightly higher rate of fluid absorption than did the

corresponding loop in the cat which received no drug. It would, therefore, seem unlikely that regional differences in response to indomethacin of the small intestine influence the ability of cholera toxin to stimulate fluid secretion in the intestine. The results confirm the observations made with terminal ileal loops in cats (Experiments 8, 9; Section 1, Part 2).

Experiment 11. The prostaglandin release from the small intestine in cats following cholera toxin challenge and the effect of anti-inflammatory drug pretreatment on this release

It did not appear likely that prostaglandins were essential to the mechanism whereby cholera toxin stimulates fluid loss in cat small intestine. Nevertheless it was important to investigate the release of prostaglandin-like material into the fluid of toxin-treated animals receiving drugs and those not on therapy as it was possible that prostaglandin production might not be depressed by the doses of drug used. If the doses were effective then there should be a difference in this output and this would be additional evidence refuting the suggestions made regarding prostaglandin participation in cholera (Bennett, 1971). On the other hand if no difference was seen it would suggest that the doses were indeed not sufficiently great and that one could not exclude prostaglandins as mediators of experimental cholera in cats. A single passage perfusion system decreased the likelihood that prostaglandin release might not be detected because of intestinal metabolism of released prostaglandins (see Experiment 3). The prostaglandin release from the small intestine in cats following cholera toxin challenge and the effect of anti-inflammatory drug pretreatment on this release was investigated.

Methods

The material used in this study was the perfusates collected in the two preceding experiments. The fluid which had been stored at -15°C was allowed to thaw at room temperature and was pooled according to the drug used, the dose and the route of administration of the drug. The material collected from the one animal that received 100 mg/kg indomethacin by intravenous injection was pooled

with those of three cats which were given 50 mg/kg indomethacin intravenously. Thus five batches of samples were obtained for both toxin-challenged and control loops. These were coded as follows: the 10 mg/kg cat samples were designated I(10 C) and I (10 T) for the control and toxin loop respectively; the 50 mg/kg samples - I (50 C) and I (50T); the 600 μ g/loop locally administered indomethacin - I (600 LC) and I (600 L T); the 25 mg/kg aspirin A (25 C) and A (25 T). The control experiment fluid was termed C ex C and C ex T for the control and toxin loop samples respectively.

Because the initial volume of the samples was so large it was more convenient to concentrate the organic matter in the fluid and eliminate as much of the inorganic salts as possible. This was achieved by using XAD-Amberlite columns. The prostaglandin content of the samples is concentrated in a small volume of organic solvent and can be extracted much more easily. The technique has been used by Davis (1971) to concentrate large volumes of prostaglandin-containing salt solutions.

Standard XAD-Amberlite columns were set up to determine the optimum system for prostaglandin recovery and concentration from salt solution used to perfuse the intestinal loops in the cat experiments.

Standard XAD-Amberlite columns

$^3\text{H-PGE}_1$ (0.2 μ c) was added to 4 litres salt solution (Pierce et al, 1971) and the pH was adjusted to pH 7.4 if necessary using citric acid or sodium bicarbonate. A long, wide bore glass column (105 cm x 2.5 cm) was packed with 275g XAD-Amberlite in ethanol. The XAD-Amberlite was washed with 200 ml ethanol and then with 3 litres distilled water. The salt solution was then poured down

the column and the flow rate was adjusted to 25 ml per minute. Once the sample had all been applied to the column, 500 ml distilled water was passed down the column. This was followed by 150 ml ethanol and then by a further 300 ml and finally by two fractions of methanol, each 200 ml volumes. The four eluate fractions were collected separately and evaporated to dryness. The residue of each was dissolved in 1 ml methanol and a 100 μ l aliquot was taken for scintillation counting. The percentage recovery of PGE in each fraction was calculated and the total recovery from the column was obtained. The results are shown in Table 18 in the results section.

The procedure was repeated using ^3H -PGA₁ (0.2 μ Ci) and similar recovery determinations were made. The results are contained in Table 18.

XAD-Amberlite Column Concentration of Fluid Samples

The procedure was essentially the same as that for the standard columns. Prior to passing the samples down the column, ^3H PGE₁ (0.2-0.3 μ Ci) was added to the bulked material for each group of cats and the pH of the sample was adjusted to pH 7.4 as before. The flow rate was 25 ml/min. and the volumes of the various solvents was the same as in the standard columns. The eluates were evaporated to dryness, redissolved in 1 ml methanol and aliquots removed for radioactivity recovery determinations. Fractions eluted ahead of the major peak of radioactivity were discarded as were all fractions containing less than 10% of the total tritium label added to the column. Those fractions which were immediately behind the main peak were also rejected unless they contained more than 10% of the label. If they contained more than 1/10 of the total radioactivity, they were pooled with the main peak and the combined

fraction was evaporated to dryness in preparation for subsequent extraction and thin layer chromatography.

Extraction of concentrated samples

The dried methanol fractions were dissolved in 25 ml 67% ethanol and partitioned with 10 ml heavy petroleum spirit (B.Pt.60-80°) and the petroleum phase was discarded. The ethanolic solution was evaporated to dryness and then dissolved in 20 ml distilled water. The pH was adjusted to pH 4.5 with citric acid and the sample was then partitioned against ethyl acetate. The ethyl acetate was washed with 4 ml distilled water and evaporated under reduced pressure.

Thin Layer Chromatography

Thin layer glass plates were prepared as described previously in Section 1, Experiment 1. The solvent systems used were the A1 system of Green and Samuelsson (1964) and the HD11 solvent system described by Davis in 1971. This latter solvent was made up of 25 parts toluene to 15 of dioxane and 0.5 parts acetic acid. The plates were allowed to run to a height of 15 cm on each of the two runs. The first solvent run was in the A1 solvent and the second was in the HD11 system. Standard prostaglandins were run on a separate plate and the position of the PGE_1 in the plates with the samples was compared to that of the standard plate PGE_1 . From this it was possible to estimate the position of the PGE_1 and the PGA_1 on the sample plates. The standard plate contained $^3\text{H-PGA}_2$ and $^3\text{H-PGE}_1$. The amount of each prostaglandin added was 0.1 μCi . The prostaglandin zones were scraped from the plates and the remainder of the plate was divided into 2 cm bands which were also scraped off the plates. All of these fractions were eluted with 2.5 ml methanol. The methanol was evaporated to dryness and the

samples were dissolved in 1 ml distilled water for fractions to be assayed against PGE_1 on the rat fundal strip or in 1 ml 0.9% saline in the case of samples to be assayed on the kitten blood pressure preparation.

Biological Assay

The preparations used to estimate the prostaglandin content of the samples were the rat fundal strip and the kitten blood pressure assay. Both of these procedures are described in Section 1, Experiment 1. The rat fundal strip was sensitive to 2.5 ng PGE_2 i.e. to a final concentration of 0.5 ng/ml PGE_2 . It was possible to detect 20-25 ng PGA_1 /kg in the kitten used.

XAD Experiments

Results. (A) XAD Chromatography.

(a) Standard XAD Columns.

Table 18. The recovery of $^3\text{H-PGE}_1$ and $^3\text{H-PGA}_1$ in eluates of XAD-Amberlite Columns.

Sample Volume 4 litres pH = 7.4

Flow Rate 25 ml/min.

Initial Volume distilled water - 500 ml.

Fraction	% Recovery in Eluates	
	PGE ₁	PGA ₁
1. 150 ml Ethanol	2.4%	0.2%
2. 300 ml Ethanol	85.3%	82.0%
3. 200 ml Methanol	0.4%	0.8%
4. 200 ml Methanol	0.3%	0.3%
Total radioactivity recovered	88.4%	83.3%

The results of the standard XAD-columns are summarized in Table 18.

The majority of the standard PGE₁ and PGA₁ was recovered in fraction 2, the 300 ml Ethanol fraction - 85.3% of the PGE₁ added appeared in this fraction. The corresponding fraction of the column run with standard PGA₁ contained 82.0% of the PGA₁ added. The total recovery from the columns was 88.4% and 83.3% for PGE₁ and PGA₁ respectively.

(b) Samples run on XAD-columns

The recoveries obtained with the samples obtained from the cat experiments (Experiments 9 and 10) are shown in Table 19.

Table 19. XAD-Column

Sample	Control Exper. loop Control Toxin		Aspirin 25mg/ kg Loop Control Toxin		Indomethacin 10mg/kg Loop Control Toxin		Indomethacin 50 mg/kg loop Control Toxin		Indomethacin 600 µg Loop Control Toxin	
Total % Recovered from Column	85.2%	74.6%	92%	80%	98.4%	96.8%	100%	84.3%	72%	73¼%
Fraction 1 % distribn. label total recovered	6.8%	4.7%	0.3%	0.3%	11.4%	1.1%	4%	3.9%	1.2%	1%
Fraction 2 % distribn.	91.1%	90%	78.7%	76.8%	86.2%	93.4%	93.9%	94.2%	80%	88.3%
Fraction 3 % distribn. total re- covered	1.2%	4.7%	14.1%	19.1%		5%	1.4%	1.3%	17%	10.7%
Fraction 4 % distribn. total re- covered	0.9%	0.6%	6.9%	3.8%	2.4%	0.5%	0.7%	0.6%	1.8%	-
Fractions used for TLC & total % in combin- ed fractions	2	2	2 & 3	2 & 3	2	2	2	2	2 & 3	2 & 3
	91.1%	90%	92.8%	96.9%	86.2%	93.4%	93.9%	94.2%	97%	99%
Actual % Recovered P.G.	77.6%	82.9%	85.4%	77.5%	85.8%	90.4%	93.9%	79.4%	69.7%	72.7%

As in the case of the standard prostaglandins, the recovery of prostaglandin-like material was high. The range of recoveries in the 300 ml Ethanol fraction was 76 to 95% of the total amount recovered from the columns as estimated from tracer amounts of PGE_1 added to the samples prior to XAD chromatography. The total recoveries varied between 72% and 100% of the total tritium label added. The samples used for further investigation contained 69% to 94% of the total 3-H- PGE_1 tracer added. The mean value was 81.5%.

B. Prostaglandin Release in Cholera Toxin-Challenged Intestinal loops and the effect of anti-inflammatory drugs on this release.

The mean prostaglandin recovery from XAD chromatography was 81.5%. Recovery from silica gel plates was normally in the region of 65%. Thus the actual recovery of prostaglandin material after both procedures was on average 53% of the total amount in the sample.

(a) Control Animals - No anti-inflammatory drug treatment.

The PGE_2 -like activity detected in the toxin-challenged loop fluid from the animals receiving no drug treatment amounted to 30 ng PGE_2 equivalents while no detectable PGA_1 activity was found by biological assay by the kitten blood pressure method of estimation. The control loop also did not appear to contain any prostaglandins of the A series but the PGE_2 -like activity amounted to 20 ng PGE_2 -equivalents. The limit of detection for PGA_1 was 90ng PGA_1 . Thus the samples contained less than 180 ng total PGA_1 -like activity.

(b) Indomethacin Treated Animals. 1/Animals receiving intravenous indomethacin 10 mg/kg, 50mg/kg and 100mg/kg

No results were obtained from the group of animals treated with indomethacin 10 mg/kg owing to the loss of the pooled sample

following thin layer chromatography.

The samples from the animal given 100 mg/kg indomethacin were pooled with the appropriate samples in the cats which had received 50 mg/kg indomethacin by intravenous injection. The prostaglandin output was depressed compared to the group of animals not given antiphlogistic agents. The toxin and non-toxin challenged loops both showed depressed levels of prostaglandin output. There was no apparent difference in the PGE_2 -like activity between these samples. The total PGE_2 output was estimated to be 5 to 10 ng PGE_2 equivalents. Again no PGA_1 activity could be detected. The limit of the assay was 90 ng PGA_1 equivalents. The sample was therefore shown to contain less than 180 ng PGA_1 equivalents.

(c) Local Indomethacin Treatment

The results obtained in this experiment were somewhat surprising. The control, toxin-challenged, non-indomethacin treated loop fluid was found to contain 125 ng PGE_2 equivalents. This was greatly in excess of the amount released into fluid in toxin treated loops in the control series of experiments (Experiment 8), reported above in Section (a). No PGA_1 -like activity was found. The indomethacin-treated loop was not observed to release either PGE_2 or PGA_1 -like material. The limits of the assay systems employed were 25 ng, in the case of PGE_2 equivalents and 180 ng PGA_1 equivalents.

(d) The Effect of Aspirin 25 mg/kg on Prostaglandin Release

Aspirin treatment did not appear to suppress the prostaglandin release in the toxin treated intestinal loop. A total of 150 ng PGE_2 equivalents was detected on the rat fundal strip in this sample. The corresponding control loop fluid contained less than 25 ng PGE_2 equivalents in total. No PGA_1 activity could be detected in either

fluid sample. The amount of PGA_1 -like activity released was less than 180 ng PGA_1 in both cases.

Conclusion

Indomethacin administered systemically in a dose of 50 mg/kg was observed to depress prostaglandin synthesis by about fifty per cent when compared to untreated control animals.

Locally applied indomethacin (600 $\mu\text{g}/\text{loop}$) markedly inhibited prostaglandin output in toxin treated loops. The depression of prostaglandin E-like output was at least 80% with this dose. Aspirin (25 mg/kg) did not abolish prostaglandin release in toxin-treated intestinal loops. Control loop synthesis was depressed however.

The significance of these results is discussed in the General Discussion of Part 2 of this Thesis.

Experiment 12. The determination of plasma levels of Indomethacin

Although the results presented so far tend not to support the hypothesis that cholera toxin effects are mediated via prostaglandins, it is important to establish the non-involvement of these hormones as conclusively as possible. Hence it was desirable to show that plasma levels of indomethacin achieved in the course of experiments were sufficient to suppress prostaglandin production in the small intestine. Marked inhibition of prostaglandin biosynthesis would, if the synthesis were an essential step in the chain of events initiated by cholera toxin and resulting in fluid secretion in cholera, be expected to be associated with a definite inhibition of the fluid output. Thus the plasma levels of indomethacin were determined in cats following the intravenous administration of single doses of 10 mg and 50 mg/kg.

Methods

(a) Animal Experiments

Three kittens (1.5 - 1.8 kg) and 2 young cats (1.2 and 2.0 kg) were anaesthetized with pentobarbitone sodium 40 mg/kg given intraperitoneally. Anaesthesia was maintained by the slow intermittent infusion of pentobarbitone sodium solution (6 mg/ml). Tracheostomy was performed. The right common carotid artery was cannulated and the blood was recorded via a Statham pressure transducer connected to a Grass polygraph. Cannulae were inserted in the right external jugular vein and one of the femoral veins for the infusion of anaesthetic solution and the injection of indomethacin respectively. The ipsilateral femoral artery was cannulated for the withdrawal of blood samples for indomethacin determinations. Indomethacin in doses of 50 mg/kg and 10 mg/kg was dissolved in 0.1 ml polyethylene glycol and the volume was made up to 2.5 ml with 0.9%

saline for slow intravenous injection via the femoral vein. Blood samples were taken at varying time intervals after the drug was injected, the first sample being withdrawn within 5 minutes of the injection. All blood samples were collected in citrate anticoagulant (pH 7.4) containing dextrose (24.5 g/l). Fluid replacement was given in the form of injections of dextrose solution (2.5 ml) after blood sampling.

(b) Extraction of Blood Samples + Indomethacin Measurement

The citrated blood was centrifuged at 2,000 rpm at 4°C for 20 minutes and the plasma was carefully pipetted off using a Pasteur pipette. The plasma volume was measured and the pH adjusted to pH 5.0 with dilute hydrochloric acid. The sample was then extracted into 25 ml heptane - 3% isoamyl alcohol mixture, by vortexing the sample together with the organic solvent for 2 minutes, centrifuging, and separating off the upper organic phase. Twenty millilitres of this heptane mixture was then vortexed with 5 ml 0.1N sodium hydroxide solution for a further five minutes. The organic material was discarded after centrifugation and 4 ml of the alcoholic solution was carefully pipetted off with a Pasteur Pipette and the fluorescence of the sample was determined using an MPF 2A spectrophotofluorimeter (activation maximum 295 mμ; fluorescence maximum 385 mμ, Slit width 6 mμ).

An alternative method was used to measure the concentration of indomethacin in samples in two experiments. In these experiments the sample was extracted into the heptane-isoamyl alcohol mixture but was then returned into 5 ml 0.1 M phosphate buffer pH 8.0. As in the previous method only 20 ml of the heptane-alcohol mixture was treated with the inorganic solution. The 20 ml of organic material was vortexed for 2 minutes with the buffer and then centrifuged.

A 4 ml aliquot of the phosphate buffer solution was removed by pipetting and 2 ml fractions were transferred to quartz cuvettes for optical density measurements on the Unicam SP800 spectrophotometer. The optical density at 265 m μ was determined.

(c) Standard Curves

Standard curves were constructed for indomethacin measurements using both the Unicam SP800 spectrophotometer and the MPF2A spectrophotofluorimeter. Known amounts of indomethacin were added to phosphate buffer (0.1 M, pH 8.0) and to 0.1N sodium hydroxide solution and fluorescence and optical density were measured. Fluorescence and optical density values were found to be proportional to the concentrations of indomethacin added over the concentration range investigated (0 μ g - 40 μ g indomethacin per ml solution).

(d) Recovery Experiments

Known amounts of indomethacin were added to untreated plasma and the samples were extracted as outlined above. The recovery using both fluorescence and optical density measurements was estimated.

Results

(a) Standard Curves

Standard curves were constructed for indomethacin in phosphate buffer using optical density measurements, and indomethacin in 0.1N sodium hydroxide solution, using fluorescence determination. The curves obtained are shown in Figures 18 and 19 respectively. These curves were used to calculate plasma levels of the drug in plasma samples obtained from cats and kittens injected intravenously with indomethacin in doses of 10 and 50 mg/kg.

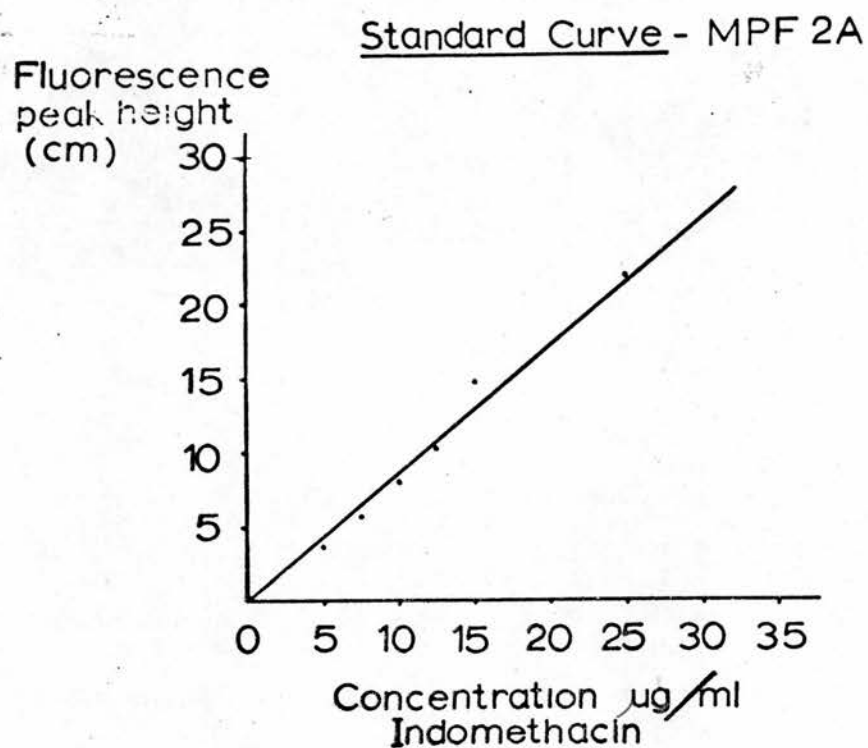


Figure 18. Standard curve of indomethacin fluorescence as determined using the MPF-2A spectrophotofluorimeter. Indomethacin concentration ($\mu\text{g/ml}$) in phosphate buffer solution is plotted against fluorescence peak height.

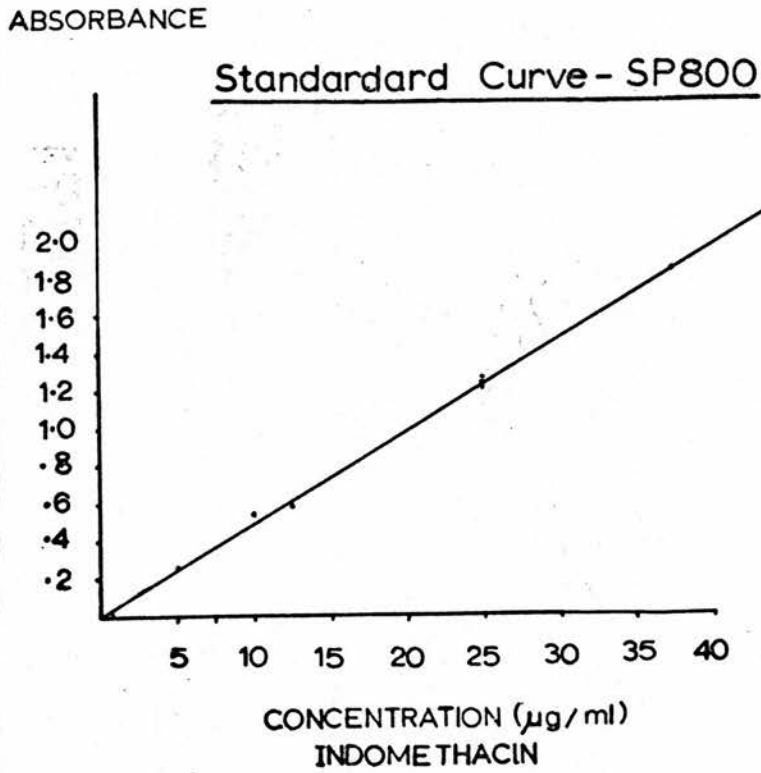


Figure 19. Standard curve of indomethacin absorbance at 265 $m\mu$ using the Unicam SP800 spectrophotometer. Indomethacin concentration $\mu\text{g/ml}$, in sodium hydroxide solution is plotted against absorbance units.

(b) Recovery Experiments

The recovery of indomethacin from plasma was found to be dependent on the concentration of the drug present in plasma to some extent. As the concentration increased, so the percentage recovery improved. In the concentration range investigated in the in vivo experiments the recovery ranged from 62.4% to 78.1%. The mean recovery was 71.5% for a concentration range of 0 to 25 $\mu\text{g/ml}$ indomethacin in plasma. With concentrations of 40 to 100 $\mu\text{g/ml}$ the mean recovery was observed to be 94.4%. These results are summarized in Table 20. The sample containing no indomethacin was used as the blank or zero value and all determinations were made relative to this.

(c) The plasma levels of indomethacin following single intravenous injections of 50 mg/kg and 10 mg/kg indomethacin.

1. The plasma levels of indomethacin in animals given 10 mg/kg drug

Three kittens received single intravenous injections of 10 mg/kg indomethacin. The plasma levels were determined over a two hour period. The plasma half life of the drug was found to be forty minutes. The peak plasma concentration was 12.5 $\mu\text{g/ml}$. The calculated peak plasma level of the drug was 13.3 $\mu\text{g/ml}$, assuming total body water distribution of the drug, total body water volume equivalent to 70% of total body mass and density of 1g/ml. The results are shown graphically in Figure 20.

2. The plasma levels of indomethacin in animals given 50 mg/kg indomethacin

Plasma levels of indomethacin were determined in two cats following the intravenous injection of a single dose of 50 mg/kg indomethacin. The peak plasma levels were observed to be 65 $\mu\text{g/ml}$ by extrapolation to zero time. The calculated plasma concentration

Table 20. The recovery of indomethacin from plasma

Sample	Indomethacin concentration added ($\mu\text{g/ml}$)	Concentration Recovered ($\mu\text{g/ml}$)	Percentage Recovery (%)
1	0	0	-
2	5	3.9 $\mu\text{g/ml}$	78.0%
3	10	7.03 $\mu\text{g/ml}$	70.3%
4	12.5	7.8 $\mu\text{g/ml}$	62.4%
5	15.0	10.6 $\mu\text{g/ml}$	70.3%
6	20.0	13.9 $\mu\text{g/ml}$	69.7%
7	25.0	19.5 $\mu\text{g/ml}$	78.1%
8	40.0	40.8 $\mu\text{g/ml}$	102.0%
9	70.0	65.5 $\mu\text{g/ml}$	93.5%
10	90.0	81.2 $\mu\text{g/ml}$	91.3%
11	100.0	90.6 $\mu\text{g/ml}$	90.6%

Mean recovery samples (2-7) : 71.5%

" " " (8-11) : 94.4%

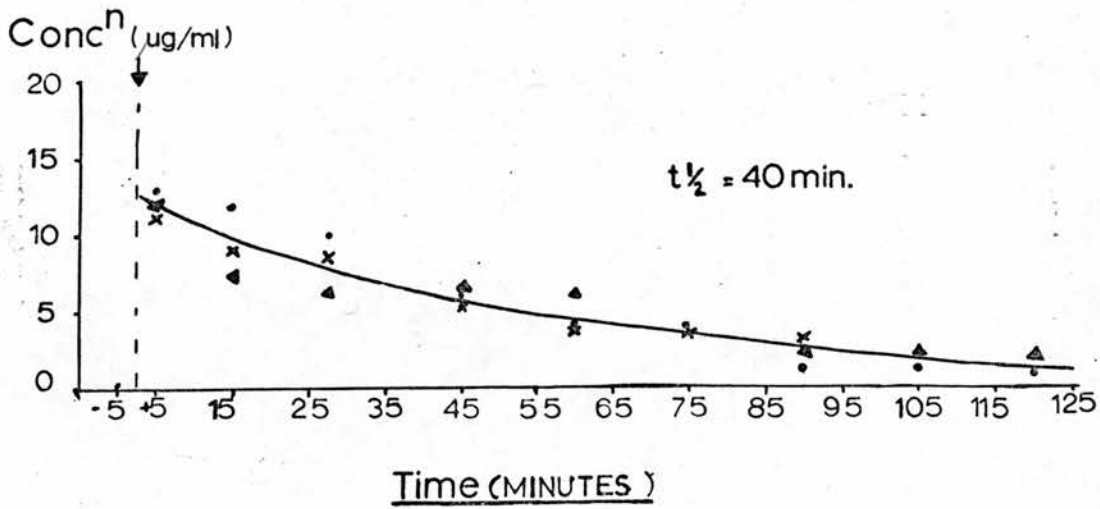


Figure 20. Plasma levels of Indomethacin following single intravenous injections of the drug in doses of 10mg/kg in three kittens.

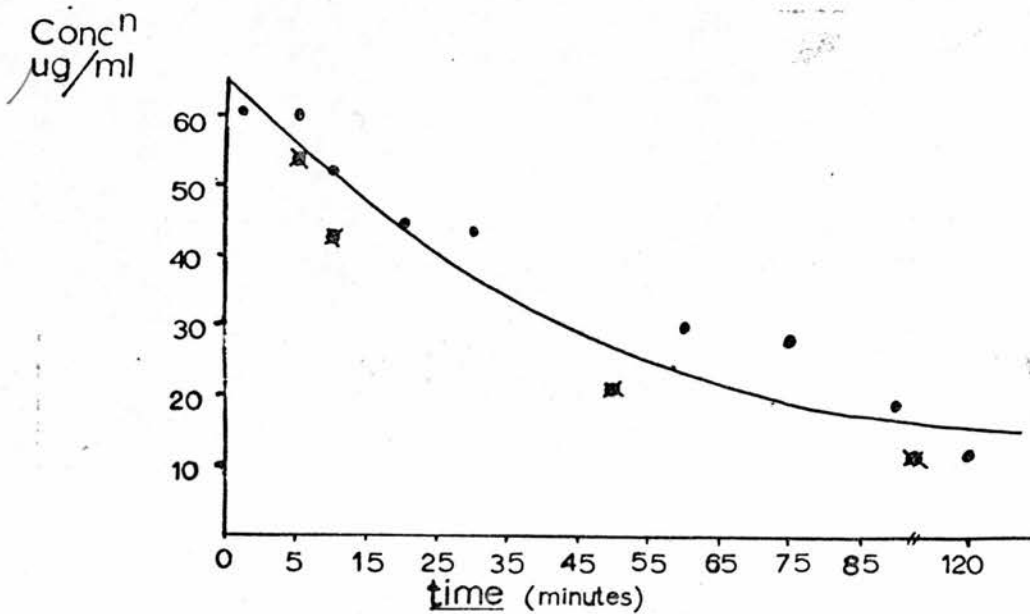


Figure 21. Plasma levels of Indomethacin following intravenous injection of 50mg/kg Indomethacin in two cats.

was 66.6 $\mu\text{g}/\text{ml}$. The plasma half life of the drug was 40-45 minutes. This showed good agreement with the results obtained in the group of animals receiving 10 mg/kg indomethacin. The results are contained in figure 21.

Discussion

The method used to extract indomethacin from plasma gave relatively good recoveries, (In the concentration range liable to be encountered in the in vivo experiments). The plasma half life of the drug is quite short but is similar to that reported by Hucker and his associates (Hucker et al, 1966) for guinea-pigs. No estimation of blood metabolites was undertaken. The extraction into heptane at pH 5.0 was found by Hucker to eliminate the necessity of such determinations (Hucker et al, 1966). The plasma levels of indomethacin during the intestinal loop perfusion experiments in the case of animals receiving 50 mg/kg indomethacin were relatively high, even after 5 hours the levels were likely to be 0.5 $\mu\text{g}/\text{ml}$. In the case of the animals receiving single doses of 10 mg/kg indomethacin the levels were much lower at this time of the order of 0.1 $\mu\text{g}/\text{ml}$ plasma. The 2 animals receiving additional doses of the drug were likely to have much higher plasma levels of the drug. With the 10 mg/kg doses given at 2 hours and 4 hours after toxin challenge the five hour plasma levels were likely to be in the region of 3-3.5 $\mu\text{g}/\text{ml}$.

Conclusion

Indomethacin has a relatively short half life in plasma, being 40-45 minutes. The levels in in vivo experiments following single intravenous injections of the drug rapidly decrease. With

single doses of 50 mg/kg the plasma levels of the drug are however appreciable after 5 hours. In animals given 10mg/kg doses, single injections result in low levels of the drug remaining in plasma at the end of a 5 hour perfusion period. Repeated dosage at 2 and 4 hourly intervals results in blood levels of the drug being maintained at a level of 3 to 3.5 μ g/ml plasma.

Experiment 13. The in vitro synthesis of prostaglandins by cat terminal small intestine and its inhibition by indomethacin

In the previous series of experiments it was found that the peak plasma levels of indomethacin achieved by intravenous doses of indomethacin (10 mg/kg, and 50 mg/kg) lay between 10 μ g and 65 μ g per ml (Experiment 12). In order to exclude the possibility that these concentrations were unable to inhibit prostaglandin synthesis and therefore resulted in the failure of indomethacin to modify cholera toxin-induced fluid secretion in the small intestine, in vitro investigations were undertaken. The in vitro synthesis of prostaglandins by cat intestinal mucosa was measured and the effect of indomethacin, 3.3 μ g/ml and 20 μ g/ml, on this synthesis was studied.

Methods

Terminal small intestine was removed from cats. The tissue was collected in ice-cold normal saline (0.9% saline). The outer muscle layers ~~were~~ carefully stripped off and discarded. The remainder of the tissue was washed carefully with saline (0.9%) and cut into small pieces with scissors, ^{and washed again}. The tissue was weighed and added to conical flasks containing ice-cold Tyrode's solution. The volume of Tyrode used was 10 ml per gram tissue. The flasks and contents were incubated at 37°C for 60 minutes in a Gallenkampf shaking water bath. The tissues were gassed with oxygen throughout the incubation period. In experiments in which indomethacin was used to inhibit prostaglandin biosynthesis, the drug was added to the Tyrode prior to the addition of the chopped mucosa. Indomethacin was prepared as a stock solution in polyethylene glycol-Tyrode solution (0.2: 6.8, v/v) containing 2.5 mg/ml indomethacin. A

1 ml aliquot was removed and the concentration of this was adjusted to 1 mg/ml with ice-cold Tyrode solution. An appropriate volume of this solution was added to the conical flasks to give final concentrations of 3.3 $\mu\text{g/ml}$ and 20 $\mu\text{g/ml}$. Incubation was then carried out as in control experiments. Prostaglandin synthesis was terminated after 1 hour by acidifying the incubates to pH 3.5 - 4.2 with 10% acetic acid and plunging the flasks and contents into an ice bath.

Extraction of Prostaglandins

The acidified supernatants were extracted three times with $1\frac{1}{2}$ volumes of ethyl acetate. The ethyl acetate phases were pooled, evaporated to a small volume (circa 20 ml), under reduced pressure, and washed with 5 ml distilled water. The washed ethyl acetate phase was evaporated to dryness and desiccated. The residue was redissolved in 25 ml 67% ethanol and this was partitioned twice with 10 ml heavy petroleum spirit (B. Pt 60-80°C). The petroleum spirit was discarded and the ethanolic phase was evaporated and desiccated. The residue was stored at -15°C overnight prior to thin layer chromatography.

Thin Layer Chromatography

Thin layer chromatography of the samples was carried out as previously described (Experiment 5, Section 1, Part 2). Zones corresponding to standard tritiated PGE_2 and $\text{PGF}_{2\alpha}$ tracers were scraped from the plates and eluted with 2 x 5 ml methanol. The methanol eluates were evaporated to dryness, desiccated and then redissolved in 1.0 ml distilled water for biological assay.

Biological Assay

Samples were assayed in terms of standard PGE_2 and $\text{PGF}_{2\alpha}$ on the rat fundal strip preparation as previously described (Experiment 5, Section 1, Part 1).

Results

(a) Control tissue synthesis of prostaglandins

The synthesis of prostaglandins of the E and F series was observed in chopped cat ileal mucosa incubated in Tyrode's solution at 37°C for one hour. The net production of PGE_2 , assayed in terms of PGE_2 equivalents on the rat fundal strip, was 347 ng/g (mean of 5 experiments), while that of $\text{PGF}_{2\alpha}$ compounds, assessed biologically in terms of authentic $\text{PGF}_{2\alpha}$, was 156 ng/g (wet weight) mucosa (mean of 5 experiments). The range of PGE_2 synthesis observed was 277-485 ng/g wet weight mucosa, whereas that for $\text{PGF}_{2\alpha}$ was much less - 126-170 ng/g wet weight mucosa.

(b) Synthesis of prostaglandins in the presence of 3.3 ml and 20 ml indomethacin

Indomethacin in concentrations of 3.3 $\mu\text{g}/\text{ml}$ and 20 $\mu\text{g}/\text{ml}$ was added to incubates of mucosal tissue. The prostaglandin output was then determined after incubation. The mean content of PGE_2 -like material fell from the control value of 347 ng to 64 ng/g wet weight tissue in samples pretreated with 3.3 $\mu\text{g}/\text{ml}$ indomethacin (2 experiments) and to 44.0 ng/g in tissues treated with 20 $\mu\text{g}/\text{ml}$ indomethacin. These drug concentrations also reduced the $\text{PGF}_{2\alpha}$ -like activity in the supernatant. The levels of $\text{PGF}_{2\alpha}$ were below the limit of detection on the rat fundal strip. The upper limit for $\text{PGF}_{2\alpha}$ in the 3.3 $\mu\text{g}/\text{ml}$ indomethacin samples was 17 ng/g wet weight

tissue and 23.0 ng/g wet weight mucosa in the 20 μ g/ml indomethacin experiments. The percentage reductions in the means were 81.5% and 89.2% for PGE₂ and PGF_{2 α} , and 87.3% and 86.3% for PGE₂ and PGF_{2 α} in the 3.3 μ g/ml and 20 μ g/ml indomethacin treated tissues respectively.

The results of these experiments are outlined in Table 21.

Conclusion

Indomethacin in doses of 3.3 μ g/ml and 20 μ g/ml is capable of inhibiting prostaglandin production by cat ileal mucosa tissue. The inhibition is marked even with the lower dose of the drug.

Table 21

Sample Weight	PGE ₂ Ng total	PGE ₂ % Recovery	PGF ₂ α ng total	PGF ₂ α % Recovery	PGE ₂ ng/g	PGF ₂ α ng/g
2.6 g	720ng	81.6%	330ng	77.8%	277.0	126
2.7 g	765ng	85.6%	440ng	84.6%	283.3	163
2.8 g	1350ng	22.8%	500ng	120.1%	485.7	160
3.3 g	1210ng	61.6%	560ng	71.5%	366.7	170
3.3 g	1035.5ng	67.6%	536ng	69.3%	313.8	162
Mean		74.1%		75.8%	347.3	156.2

Sample Weight	PGE ₂ Ng total	PGE ₂ % Recovery	PGF ₂ α ng total	PGF ₂ α % Recovery	PGE ₂ ng/g	PGF ₂ α ng/g
Ind. 2.8g	215ng	70.7%	50ng	79.0%	76.8	18ng
3.3 2.8g	145ng	73.3%	45ng	85.4%	51.8	16ng
Ind. 2.5g	120ng	106.1%	60ng	63.6%	48	24ng
20 2.5g	100ng	99.6%	55ng	70.7%	40	22ng
Mean						
2.8 Ind. 3.3	180ng	87.4%	47.5	74.7%	64.3	17.0ng
2.5 Ind. 20	110ng		57.5		44.0	23.0ng

Ind. 3.3 μg/ml 81.5% inhibn. PGE₂ & 89.2% F₂α
 20.0 μg/ml 87.3% " " & 86.3% F₂α synthesis.

General Conclusion

Little evidence was found to support the hypothesis that cholera toxin stimulated fluid and electrolyte secretion in the terminal ileum in rabbits and cats via a mechanism involving an obligatory prostaglandin mediated step.

Prostaglandins of the E and A series were detected in purified extracts of fluid aspirated from the ligated intestinal loops in rabbits but there was a marked correlation between the amounts of PGE-like and PGA-like material released and the extent of tissue damage in the intestinal wall.

Indomethacin administered prophylactically to rabbits in doses of 10 to 40 mg did not inhibit the secretory response of the ileum to cholera toxin but did result in a diminished prostaglandin release. This coincided with the absence of haemorrhagic necrosis in the ligated loops at 24 hours after toxin administration. In untreated animals haemorrhagic necrosis was invariably present at this point (Experiments 1, 2). It was therefore concluded that indomethacin prevented the onset of pathological, necrotic changes in the intestinal wall which were associated with prostaglandin biosynthesis but did not interfere with cholera toxin-induced fluid output in the rabbit ileum.

The residual prostaglandin release was not attributable to the bacterial toxin itself; no prostaglandin-like material corresponding to prostaglandins of the E, F or A series being detected in extracts of crude toxin (Experiment 1a). Horton and Collee (unpublished observations) were also unable to detect prostaglandins by biological assay of purified extracts of *V. cholerae*

12r (Ogawa) toxin.

A further reduction in prostaglandin release was observed when the perfused ileal loop model was substituted for the less sophisticated ligated loop model (Experiment 3). No prostaglandin-like activity was detected in intestinal perfusates following challenge of the intestinal mucosa with purified cholera toxin prepared from *V. cholerae* 225B or with crude toxin elaborated by *V. cholerae* 12r (Ogawa). It was evident that prostaglandin release was due to distension of the intestinal wall by fluid which accumulated in the ligated intestinal loop in experiments of short duration (8-12 hours) and that the enhanced prostaglandin output observed at 22-24 hours after toxin administration was a reflection of the degree of haemorrhagic necrosis experienced by the intestinal wall.

These conclusions were further supported by experiments in which local application of indomethacin was used in order to prevent prostaglandin biosynthesis. Large doses of indomethacin (3 mg per loop) did not inhibit the secretory response of the mucosa to cholera toxin (Experiment 4).

In vitro studies confirmed these observations. Incubation of chopped ileal tissue with purified *V. cholerae* 225B toxin did not enhance prostaglandin biosynthesis in this tissue. No increase in the PGE or PGF content of the incubate supernatant was detected by biological assay of purified extracts on the rat fundal strip (Experiment 5). Indomethacin in doses of 10 μ g and 15 μ g/ml was equally effective in suppressing prostaglandin synthesis in cholera toxin treated intestine and control, untreated tissue. It was therefore concluded that neither in vitro nor in vivo exposure of rabbit intestinal mucosa to

cholera toxin stimulates the production of prostaglandin compounds.

These results indicate that prostaglandins are unlikely to be mediators of the fluid secretion initiated by cholera toxin in rabbit experimental models of cholera.

Subsequent experiments conducted in cats confirmed these findings in this species also. The terminal ileum of young cats and kittens respond to cholera toxin challenge by secreting fluid and electrolytes. The mean volumes secreted ranged from 4 to 6 ml per 20 cm per hour. Unchallenged loops reabsorbed between 5 and 8 ml fluid in this period (Experiment 8). Indomethacin in doses of 10 to 100 mg per kg body weight given intravenously, and in a dose of 600 μ g per loop administered to the loop itself, and aspirin 25 mg per kg also given by intravenous injection did not depress the secretory activity or reabsorptive capacity of the toxin-treated and control loops respectively (Experiment 9). The release of prostaglandin-like material into the perfusates collected in the course of these experiments was estimated. It was observed that indomethacin given both locally and systemically depressed prostaglandin output between 50 to 80%. Aspirin 25 mg/kg did not abolish the release of prostaglandin-like material in toxin-challenged loops. As the fluid secretion was not impaired even although the prostaglandin output was markedly affected following administration of the prostaglandin synthesis inhibitor, indomethacin, it is clearly demonstrated that in vivo, cholera toxin does not operate ^{primarily} through a prostaglandin-like mediator when inducing fluid secretion.

Experiments were also performed using sections of jejunum in cats. As in the ileal region of the intestine it was not possible to inhibit fluid secretion in toxin-challenged loops by

systemic pretreatment with indomethacin in a dose of 10 mg/kg body weight (Experiment 10).

The plasma levels of indomethacin were measured following single intravenous injection of doses of 10 mg and 50 mg/kg body weight. The peak plasma levels were 13 μ g and 65 μ g per millilitre respectively. The plasma half life was also determined and was found to be 40 to 45 minutes (Experiment 12).

It was further demonstrated that in vitro prostaglandin biosynthesis could be markedly inhibited on incubation of chopped intestine with concentrations of indomethacin as low as 3.3 μ g per ml. At this dose level inhibition of PGE-like synthesis was 81.5% while the corresponding value for PGF-like compounds was 89.2%. Levels of 3.3 μ g per ml and greater were maintained for 90 minutes following single indomethacin injections of 10 mg/kg, and for the entire experiment where multiple injections of indomethacin were employed. The single 50 mg per kg dose ensured that plasma levels in excess of 3.3 μ g per ml were maintained for at least 3 hours. Thus it was clearly demonstrated that plasma levels of indomethacin encountered in in vivo experiments were capable of significantly reducing prostaglandin production by intestinal tissue.

In summary, it was clearly demonstrated that cholera toxin did not cause fluid secretion in the small intestine of either cats or rabbits by stimulating prostaglandin production. Three lines of approach were used: firstly, the in vivo release of prostaglandins in ligated and perfused rabbit intestinal loops and cat perfused loops was examined; secondly, the effect of local and systemic administration of a potent prostaglandin biosynthesis inhibitor, indomethacin, in fluid production and prostaglandin release

was studied; thirdly the in vitro biosynthesis of prostaglandins and the effect of toxin and indomethacin on this synthesis was determined. All the evidence obtained indicated that cholera toxin did not stimulate prostaglandin synthesis and that treatment with prostaglandin synthesis inhibitors did not suppress the secretory response of intestinal tissue to cholera toxin, at dose levels which markedly inhibited prostaglandin biosynthesis.

Experiment 1. Section 2

The prostaglandin content of E. coli crude heat stable enterotoxin prepared from E. coli strain B44

As in the case of experiments performed using crude toxin material prepared from *V. cholera*^e, it was necessary to eliminate a contribution from *E. coli* enterotoxin itself to the prostaglandin content of intestinal fluid and supernatant in tissue incubation studies. The prostaglandin content of crude *E. coli* enterotoxin used to infect piglets and calves was therefore investigated.

Methods

E. coli Strain B44 heat-stable, crude enterotoxin was supplied by Dr. R. Bywater (Royal (Dick) Veterinary School, Edinburgh). 60 ml volumes of the toxin were subjected to prostaglandin extraction procedures. Three batches of B44 toxin, prepared on separate occasions were used. The techniques used for extraction were those previously described in Experiment 1, Section 1, Part 2. Method B, Figure 6b was used. Standard tracer tritiated PGE₂ and PGA₂ were added to the sample prior to extraction. 0.1 μ Ci of each was added. Control sterile broth was also subjected to the extraction procedure.

Thin Layer Chromatography

Residues obtained after extraction of the samples of enterotoxin were subjected to thin layer chromatography. A double solvent run development procedure was used. The solvent systems used were the F VI solvent system for the initial run and GCM solvent system for the subsequent development of the samples. The 67% ethanol residue was applied as a narrow band to the ready prepared Merck 20 x 5 cm plates coated with a layer of Silica gel G 0.25 mm thick. The plates were then run in the solvent systems described and the solvent

front in both cases was 15 cm from the origin. Before the plates were developed for the second time the origin zone of the first run was scraped off and the plates were then run from the new baseline. The removal of the origin zone ensured that the majority of the pigment contained in the extract which was retained at the origin, was eliminated and this gave much cleaner samples than would otherwise have been obtained. Standard tracer $\text{PGF}_{2\alpha}$ was incorporated into the samples prior to thin layer chromatography and thus the appropriate prostaglandin zones could be scraped off the plates and eluted with methanol. The scraped zones were vortexed with 5 ml methanol and centrifuged. The supernatant was decanted off. This procedure was carried out twice and the methanol eluates were pooled, evaporated to dryness and desiccated. The samples were then dissolved in 1 ml methanol. A 0.1^{ml} aliquot was used for prostaglandin recovery estimations and Biological Assay was performed on the remaining 0.9 ml of the sample.

Recovery Determination

The 0.1 ml aliquots of the methanol samples obtained after thin layer chromatography were added to 10 ml toluene scintillant. The radioactive content of the samples was determined by scintillation counting using a Nuclear Chicago scintillation counter. The percentage recovery of the PGE_2 , $\text{PGF}_{2\alpha}$ and PGA_2 activity added ($0.1 \mu\text{Ci}$ of each had been added) was determined.

Biological Assay

(a) Biological assay of the PGE_2 and $\text{PGF}_{2\alpha}$ zones obtained after TLC using the rat fundal strip allowed estimations of the PGE_2 -like and $\text{PGF}_{2\alpha}$ -like content of the enterotoxin extract to be made. Standard solutions of PGE_2 and $\text{PGF}_{2\alpha}$ were used. The preparation was sensitive to $0.5 \text{ ng/ml } \text{PGE}_2$ and $3 \text{ ng/ml } \text{PGF}_{2\alpha}$. The technique is

described in Section 1, Part 2, Experiment 5.

(b) The kitten blood pressure assay preparation was used to estimate the PGA content of crude enterotoxin. The assay had a lower limit of sensitivity of 30 ng PGA_1 in the animal used on this occasion. This was equivalent to 25 ng PGA_1/kg body weight.

Results

(a) Recovery of Exogenous Prostaglandins

The percentage recovery of $\text{PGF}_{2\alpha}$ activity added to the samples prior to TLC was 85-88% while that of PGE_2 was 57-67%. PGA_2 recovery was 65 - 72%. The higher recovery of $\text{PGF}_{2\alpha}$ is partly due to the addition of this compound immediately prior to thin layer chromatography and not, as in the case of the PGE_2 and PGA_2 , before beginning the sample extraction procedure.

(b) Prostaglandin Content of E. coli toxin

In two of the three samples no prostaglandin-like activity was detected either on the rat fundal strip or on the kitten blood pressure preparation. In the third sample 10 ng PGE_2 -like activity and 100 ng $\text{PGF}_{2\alpha}$ -like activity was detected. In this sample no PGA_1 -like activity was present. The limit of detection of PGE_2 was 10 ng PGE_2 , that of $\text{PGF}_{2\alpha}$ was 25 ng and 60 ng PGA_1 per 60 ml for all of the three samples of toxin solution.

(c) Prostaglandin Content of Sterile Broth

As in the case of the toxin samples, no prostaglandin-like activity was detected in 2 of the 3 sterile broth samples. These corresponded to the enterotoxin batches in which no prostaglandin activity was detected. In the third 12 ng PGE_2 and 100 ng $\text{PGF}_{2\alpha}$ activity was found. No PGA_1 -like activity was

found in any of the three samples. The limit of detection was 60 ng PGA_1 . The sample with PGE_2 and $\text{PGF}_{2\alpha}$ -like activity was prepared at the same time as the E. coli toxin batch which contained prostaglandin-like activity. The PGE_2 activity per ml was 0.2 ng of PGE_2 and 1.7 ng/ml of $\text{PGF}_{2\alpha}$ in the toxin and sterile broth which were biologically active.

Discussion

Two of the three batches of toxin and sterile broth contained no detectable prostaglandins of either the E, F or A series. The third batch of sterile broth and toxin contained PGE and F-like activity. The PGE activity in the sterile broth was marginally greater than that of the toxin, while there was no difference between the PGF content of the two.

The reason for the activity in this third sample of sterile broth is not clear. It is curious that the activity in the toxin prepared using that sterile broth contained an equal amount of prostaglandin-like activity. It therefore suggests that some contaminant was present, which was carried through the extraction, purification and separation techniques. The result of the third batch of toxin and sterile broth does not contradict the findings of the other two experiments as the prostaglandin-like activity contained in the toxin solution was no greater than that in the sterile broth used to prepare toxin solutions.

Conclusion

The prostaglandin content of E. coli toxin prepared from strain B44 is no greater than that of sterile broth. Sterile broth is unable to stimulate fluid production in the small

intestine of piglets or calves. Therefore the possession of prostaglandin-like activity by the toxin is unlikely to be responsible for the fluid losses observed in *E. coli* induced diarrhoea. The contribution made to the prostaglandin content of fluid secreted by the small intestine following *E. coli* enterotoxin administration is negligible. The maximum contribution was 0.2 ng PGE_2 , 1.7 ng $\text{PGF}_{2\alpha}$ and 1 ng PGA_1 -like activity when 1 ml toxin was used for challenge.

The prostaglandin content of fluid washings from healthy and E. coli-infected piglets

As in cholera, E. coli infection results in diarrhoea. The animals selected for this study were neonatal piglets which were infected with E. coli heat-stable enterotoxin. Intestinal washings samples were supplied by Dr. R. Bywater of the Royal (Dick) Veterinary School, Edinburgh University. Intestinal washings were also obtained from healthy, non-infected control piglets. The prostaglandins present in washings from both healthy and infected animals were investigated.

Methods

Samples were collected and transferred on ice from the Royal (Dick) Veterinary School to the Department of Pharmacology, and were either extracted immediately or stored at -15°C overnight, when extraction was performed the following day.

Extraction

The technique used to extract the prostaglandin-like material in the samples was that outlined in Figure 6B, Experiment 2, Section 1, Part 2 : Method B. The 67% ethanol residue was then subjected to thin layer chromatography. Radioactive tracer 3-H-PGE_2 , $\text{PGF}_{2\alpha}$ and PGA_2 , 0.1 μCi of each was added to the samples prior to extraction. The amount of non-radioactive prostaglandin added was less than 4 ng.

Thin Layer Chromatography

Thin layer chromatography procedure was as described in Experiment 1, Section 2: The methanol eluates of the PGE , PGF , and PGA containing zones were evaporated to dryness and redissolved in 1 ml methanol. At this point the samples were split: 0.1 ml was used to determine the percentage recovery of prostaglandin-like

material in the sample by scintillation counting techniques;

0.9 ml sample was used for biological assay.

Scintillation Counting and Recovery Determinations

These procedures have been described previously in Experiment 1, Section 2.

Biological Assay

Samples were assayed using the rat fundal strip and kitten blood pressure preparation as in Experiment 1, Section 2.

Results

The results of both control and infected animals are contained in Table 21A.

(a) Uninfected Animal Intestinal Washings

Samples were obtained from 2 animals. The samples contained 3.3 ng and 0.9 ng per ml PGE_2 -like activity. The $\text{PGF}_{2\alpha}$ -like content was 5.7 ng/ml and 7.3 ng/ml in these samples. No PGA-like activity was detected. The limit of detection of 50 ng total and 60 ng total respectively for these samples which corresponded to limits of 2.5 ng and 16 ng per ml for the respective samples. The average PGE_2 output was 2.1 ng/ml and that of $\text{PGF}_{2\alpha}$ was 6.5 ng/ml.

(b) Infected Animals' Intestinal Washings

Four samples were obtained from infected animals. The prostaglandins detected were PGE and PGF series compounds. As in the uninfected animals no PGA-like activity was found. The mean PGE_2 output was 4.0 ng/ml and the range of values was 1.8 ng/ml - 8.0 ng/ml. In two samples no $\text{PGF}_{2\alpha}$ -like activity was found. The limits of detection for these samples were 1.9 ng/ml and 7.3 ng/ml. In the two remaining samples the $\text{PGF}_{2\alpha}$ concentrations were observed to be 12.1 ng/ml and 4.0 ng/ml. The mean output, using the limits of

Table 21A. The prostaglandin content of intestinal washings from healthy and infected piglets

Sample and volume (ml)	Prostaglandin Zone	Percentage Recovery	Prostaglandin Content-Total ng	Prostaglandin Concentration ng/ml
Control 19.0 ml	PGF _{2α}	85.4%	110ng	5.7 ng/ml
Control 3.7 ml	PGF _{2α}	61.4%	26ng	7.3 ng/ml
Average values		73.4%		6.5 ng/ml
Control 19.0 ml	PGE ₂	65.1%	61.5ng	3.3 ng/ml
Control 3.7 ml	PGE ₂	(25.0%) (65%)	3.2ng	0.9 ng/ml
Average values		45.1%		2.1 ng/ml
Control 19.0 ml	PGA ₂	69.1%	50ng	2.5 ng/ml
Control 3.7 ml	PGA ₂	130.7%	60ng	16.0 ng/ml
Average values		99.7%		9.3 ng/ml
Toxin 13.0 ml	PGF _{2α}	74.2	25	1.9 ng/ml
Toxin 4.0 ml	PGF _{2α}	76.7	48.2	12.1 ng/ml
Toxin 6.8 ml	PGF _{2α}	88.8	50	7.3 ng/ml
Toxin 10.0 ml	PGF _{2α}		40	4 ng/ml
Average values				6.3 ng/ml
Toxin 13.0 ml	PGE ₂	87.6	23	1.8 ng/ml
Toxin 4.0 ml	PGE ₂	(17.6) (57.6)	11.7	2.9 ng/ml
Toxin 6.8 ml	PGE ₂	84.2	55	8.0 ng/ml
Toxin 10.0 ml	PGE ₂		33	3.3 ng/ml
Average values				4.0 ng/ml
	PGA ₂ none detected		65ng	1.9 ng/ml

sensitivity as the output in samples where no prostaglandin was detected, was 6.3 ng/ml.

Discussion

The $\text{PGF}_{2\alpha}$ -like output by control, non-infected and infected animals did not differ significantly. The PGE_2 output mean figures do show a higher mean value for the infected animals than in the two non-infected animal average. The range of values in both cases was large and statistical analysis showed that the difference was not significant ($p > 5\%$). The student 't' test was used for this evaluation. No conclusion about the PGA output could be made by the assay used. It is possible that with more sensitive techniques, for example radio-immunoassay, that PGA output might be detected. The recoveries of PGE in the 3.7 ml control sample and 4.0 ml toxin sample were unusually low but the PGA recovery was remarkably high, in both cases exceeding 100%. It was found that the tracer prostaglandin E had in fact deteriorated and approximately 45-50% of it had been converted to PGA_2 . In the determination of PGE_2 and PGA_2 levels in both of these samples, this factor was allowed for. The standard PGE_2 solution was purified by Sephadex LH-20 chromatography by C. Hensby and on thin layer chromatography of the PGE eluate from the column, only one peak of activity was detected. This material was then eluted from the thin layer plate and used as standard tracer PGE_2 in subsequent experiments. It was 99.9% pure.

Conclusion

Intestinal washings from control and toxin infected animals do not appear to differ in their prostaglandin content. Thus *E. coli*

enterotoxin does not appear to enhance prostaglandin release from the small intestine.

Experiment 3

Prostaglandin production by healthy and E. coli enterotoxin infected intestinal tissues

Small intestine from healthy and infected piglets was removed during experiments by Dr. R. Bywater. The tissue was immersed in the cold Tyrode and transferred on ice. All samples were kept on ice as far as was possible prior to incubation. The tissue was freed from any mesentery fat and blood vessels, dried with blotting paper and weighed. It was chopped with scissors and then homogenised in 25 ml ice cold Tyrode solution using a Waring blender, at maximum speed for 2 minutes. The homogeniser tube was surrounded by a jacket of ice during the homogenization process. The homogenate was then transferred to conical flasks and the volume adjusted so that the weight to volume ratio of the tissue to Tyrode solution was 1:10 - (g:ml). The flask and contents were then incubated at 37°C in a Gallenkampf shaking water bath for 45 minutes and the tissue was gassed with oxygen during the incubation period. The reaction was terminated by plunging the conical flasks containing the intestinal homogenate into ice baths and acidifying the incubate to pH 4.0 - 4.5 with 10% acetic acid.

Extraction

Standard $^3\text{-H-PGE}_2$, $^3\text{-H-PGF}_{2\alpha}$ and $^3\text{-H-PGA}_2$, 0.1 μCi of each, were added to the acidified material. The amount of non-radioactive prostaglandin contained in these radioactive tracers was less than 4 ng in the 100 μl aliquot added to the samples. The samples were then extracted using Method B, Section 1, Part 2, Experiment 2. The 67% ethanolic residue was subjected to thin layer chromatography.

Thin Layer Chromatography

Thin layer chromatographic separation of the prostaglandin F,

E and A-like activity in the extracts was performed by using the double development technique using FVI solvent system in the initial run and the GCM solvent system in the second solvent development of the samples, as described previously (Experiment 5, Section 1, Part 2). The zones corresponding to the PGE, F and A standards were scraped from the plates and eluted with 2 x 5 ml methanol. The methanol fractions were pooled, evaporated to dryness and redissolved in 1 ml methanol. Aliquots were removed for recovery estimations (0.1 ml), for Gas Chromatography-Mass Spectrometry analysis (0.4 ml) and the remainder (0.5 ml) was used for Biological Assay.

Biological Assay

The PGE and PGF fractions of samples were assayed using the rat fundal strip preparation (Experiment 5, Section 1, Part 2) while the PGA content of the eluates from thin layer chromatography PGA zones were estimated using the kitten blood pressure preparation (Experiment 5, Section 1, Part 2).

Recovery Estimations

The recovery of exogenous prostaglandins was determined by calculating the percentage recovery of tritium labelled prostaglandins added to the samples prior to extraction. Scintillation counting techniques were employed. The technique is described in Experiment 1, Section, 2. This gave an indication of the recovery of endogenous prostaglandin in the samples.

Gas-Chromatography-Mass Spectrometry

(a) PGF_{2α} Samples

The 0.4 ml aliquot from the thin layer plates was evaporated to dryness in a 10 ml glass, pear shaped flask. Deuterium carrier D₄-PGF_{2α} (5 µg) was added to each of the samples. This was

sufficient for 5 injections. The biological extracts and deuterium carrier prostaglandin were methylated in the pear shaped flask by adding excess of freshly prepared diazomethane in diethyl ether methanol (9:1) and allowing it to react for 15 minutes. The solvent was evaporated off with a fine jet of air and the residue was desiccated by evacuation at reduced pressure. The sample was then dissolved in 0.5 ml methanol and transferred to an Eppendorf tube and the original flask was washed with 2 x 0.25 ml aliquots of methanol which were also added to the Eppendorf tube. The solution was evaporated down with a fine jet of air before and between each addition of methanol washings, and was finally evaporated to dryness and desiccated. A 50 μ l volume of bis-(trimethylsilyl)-trifluoroacetamide (BSTFA)-Sigma Chemicals - was added to each sample and these were then allowed to stand at room temperature overnight. 10 μ l amounts of each sample were injected on to the gas chromatographic column without removal of the BSTFA.

Analysis was performed using an LKB 9000 gas chromatograph-mass spectrometer. The column (3.0m x 1.5 mm i.d.) was packed with 3% OVI on a Supelcoport pretreated with dimethylchlorosilane. The column temperature was 240-260°C. The carrier gas was helium and the flow rate was 30 ml per minute. The mass spectra were recorded at an electron voltage of 27.5 mV and 22.5 mV.

Mass spectra of authentic PGF_{2 α} prepared as the methyl ester/trimethylsilyl trifluoroacetamide (Me/TMS) derivative in the same manner as the samples, were taken. Mass spectra of the samples were taken at the appropriate retention time.

(b) PGE₂ and PGA₂ samples

The PGA₂ and PGE₂ samples reserved for gas chromatography-mass spectrometry were treated with 0.5 ml 0.1N potassium

hydroxide in methanol. The samples were allowed to stand for 1 hour at room temperature. The PGE_2 samples dehydrate to the corresponding PGA compound under these conditions and this then isomerizes into the more stable PGB conformation.

After the reaction was thought to have gone to completion, 20 ml distilled water ~~were~~ added to the flasks containing the samples and the acidity was adjusted to pH 4.0 with hydrochloric acid. The samples were then partitioned with 40 ml ethyl acetate twice. The ethyl acetate phases were pooled and evaporated to dryness at reduced pressure. The residue was desiccated and then redissolved in 0.5 ml methanol and transferred to clean, dry Eppendorf tubes. The original flasks were rinsed with 0.25 ml methanol two times and the washings were added to the appropriate samples. The solvent was vaporized off with a fine jet of air and the residue was desiccated. The Me/TMS derivative was prepared as described in the previous section as for $\text{PGF}_{2\alpha}$ samples. The Me/TMS derivative of 1 μg authentic PGB_2 was prepared and this was injected on to the column, and the retention time was noted. The Me/TMS derivative of the extracted material was then injected on to the column and mass spectra were taken at the appropriate retention time for PGB_2 .

Results

(a) Biological Assay

The results of the biological assay are summarized in Table 21B. The intestinal homogenates from healthy and infected piglets were observed to synthesize prostaglandins of the F and the E series. No PGA-like activity was detectable in any of the samples. The limit of the assay was 90 ng PGA_1 -like activity for each sample. The mean production of $\text{PGF}_{2\alpha}$ -like material by healthy piglets was 108ng/g

Table 21B. The synthesis of prostaglandins of the F and E series by pig small intestine taken from healthy and Escherischia coli infected piglets, in vitro. '!' The presence of PGF_{2α} was confirmed in these samples by combined gas chromatography-mass spectrometry

Sample & Weight (g)	PGE ₂ μg total	PGE ₂ ng/g	PGF _{2α} ng total	PGF _{2α} ng/g
Control 5.2 g	1.6 μg	307.7 ng/g	460 ng *	88.5 ng
Control 4.3 g	1.53 μg	355.8 ng/g	550 ng *	127.9 ng
Control 3.2 g	1.2 μg	363.6 ng/g	-	-
Control 11.3 g	4.8 μg	424.8 ng/g	-	-
MEAN Control		363 ng/g		108.2 ng/g
Infected 3.3 g	0.8 μg	242 ng/g	400 ng *	121.2 ng/g
Infected 3.2 g	1.33 μg	415 ng/g	500 ng *	156.2 ng/g
Infected 9.3 g	3.2 μg	344.0 ng/g	-	-
Infected 7.3 ng	1.6 μg	219.3 ng/g	-	-
MEAN		328.7 ng/g		138.7 ng/g

while that of infected intestinal tissue was 139 ng/g. The difference was not statistically significant ($p > 5\%$) when assessed by the paired student 't' test method. The mean rate of PGE_2 synthesis was 363.0 ng/g in healthy intestinal homogenates and 328 ng/g in homogenates from E. coli-infected piglets. The difference between the rates of synthesis was not statistically significant ($p > 5\%$).

(b) Gas Chromatography-Mass Spectrometry

The presence of $\text{PGF}_{2\alpha}$ in extracts from E. coli-infected and healthy piglet homogenates was confirmed by gas chromatography-mass spectrometry. The ratio of protium to deuterium peaks were greater in the biological extracts than in the deuterated prostaglandins alone, thereby indicating the presence of $\text{PGF}_{2\alpha}$ in the samples. Peaks were present in the mass spectra of the samples at the following m/e values - 584, 569, 513, 494, 423 and 397 and it was the ratio of these peaks that was used to determine whether $\text{PGF}_{2\alpha}$ was present or not. The line diagram for one of these samples is shown in figure 22.

The presence of PGE_2 and PGA_2 could not be confirmed by mass spectrometry owing to the lack of purity of the samples. It was impossible to distinguish the peaks corresponding to standard PGB_2 in the spectra of the samples from the background contaminants and dirt.

Conclusion

The synthesis of $\text{PGF}_{2\alpha}$ in toxin-treated and healthy piglet intestine were not significantly different. The synthesis of PGE_2 was also not observed to differ in the tissue exposed to E. coli toxin and uninfected intestine. No PGA_2 synthesis was observed in either healthy or infected intestine. The presence of $\text{PGF}_{2\alpha}$ was confirmed by gas chromatography-mass spectrometry.

Experiment 4

The effect of prostaglandins on water, glucose and electrolyte movement in the small intestine

From the in vitro studies of prostaglandin synthesis in *E. coli* with infected and healthy piglet intestine and the lack of significant enhancement of prostaglandin output in intestinal washings, it did not seem likely that prostaglandins were involved in the *E. coli*-induced diarrhoea (Experiments 2 and 3, Section 2). The evidence was however by no means conclusive. More direct attempts were made to investigate the possible role of prostaglandins in the secretory mechanisms initiated by enterotoxin. If prostaglandins are indeed mediators of the diarrhoea, then these compounds should be able to stimulate fluid production by the intestinal mucosa. This approach was attempted with a series of 9 animals. Prostaglandins of the E and A series were employed.

Methods

Nine piglets were anaesthetized with pentobarbitone sodium injected into the anterior vena cava. A dose of 25 mg/kg was used. A midline incision was made and the intestine was carefully withdrawn from the abdominal cavity. Loops of approximately 10 cm in length were made in the upper, middle and small intestine and were cannulated with soft multiperforate cannulae. The loops were then washed with warmed 0.9% saline solution.

The animals were divided into the following experimental groups.

(a) Group (A). There were four piglets in this group. In these animals the intestinal loop was challenged with PGA_1 after the first control period. PGE_1 was given after the second control observations (2 animals) or at 50 minutes after PGE_1 measurements had been made (2 animals).

Group B. This group was made up of three piglets. Each piglet was challenged with PGE_1 intraluminally after the initial control period and subsequently with PGA_1 after the second control measurements were taken.

Group C. Of the two animals in this group one animal was treated with only PGE_1 and the other received PGA_1 intraluminally. Two prostaglandin "test" periods were studied in these animals.

The experimental procedure used was the same, irrespective of the group of animals. Test solution similar to that described by Bywater (1973) was prepared and contained sodium chloride (4.772 g/l), sodium bicarbonate (2.185 g/l), potassium chloride (0.2985 g/l), phenol red 50 $\mu\text{g}/\text{ml}$, glucose (10g/l), ^{22}Na (2 $\mu\text{Ci}/\text{l}$) and D_2O (1:100 w/v). A 1.5 ml volume of this was injected into the washed loop and the initial sample was taken to be equivalent to this test solution in composition as the volume injected was too small to allow for initial samples to be taken as in previous calf experiments (Bywater, 1973; Thesis Section 2, Experiment 6). The test solution was allowed to remain in contact with the intestinal mucosa for 10 minutes. The loops were then drained of fluid. PGE_1 or PGA_1 in doses of 20 μg and 40 μg per loop were then injected into each loop. The prostaglandin was dissolved in test solution in both cases. Prostaglandin A test solution contained 40 μg PGA_1 per 1.5 ml volume while the prostaglandin E test solution contained 20 μg PGE_1 in 1.5 ml. The loops were drained after ten minutes and a forty minute recovery period ensued. A second control observation was made in 3 animals, in the remaining 6 animals the second prostaglandin was injected in the appropriate test solution and a further ten minute intestinal absorption pattern was determined in each piglet. In animals which

were subjected to an intermediate control observation, the second prostaglandin was administered immediately after these control readings were taken. The prostaglandin given was in accordance with the experimental schedule outlined above. Again the absorption values were determined after a ten minute mucosal contact time. A further forty minute recovery period was allowed to elapse and the final control study was made.

Aliquots of the samples collected from each loop were removed for water, sodium, chloride, potassium and bicarbonate net flux determinations.

The techniques used to determine sodium and water fluxes were those described by Bywater for ligated calf intestinal loops (Bywater 1973b). Net movement was obtained by determining the difference between the insorption and exsorption values.

Bicarbonate determinations were by microdiffusion as described in Conway (1962) - Microdiffusion Analysis and Volumetric Error, 4 edition, Crosley Lockwood.

Chloride ion determinations were performed using an EEL chloridimeter and potassium was estimated by flame photometry.

Results

There was no apparent difference in the effect seen with prostaglandin E compounds injected after the first control period and after the forty minute recovery period. The same was also true for PGA injections. The results of all the PGE and PGA treated loops were therefore pooled separately. The results are summarized in Figures 23a - f and are tabulated in Tables 22a - f.

(a) The Effect of Prostaglandin on Net Water Movement

The results are contained in Figure 23a and Table 22a. Negative values indicate reabsorption and positive values secretion of water. In all of these loops the initial control value is somewhat exaggerated, this being due to the blood leakage which occurs and interferes with the phenol red estimation used to calculate the water volume. The upper and middle intestine does however appear to show a secretory pattern when challenged with PGA_1 . The position of PGE_1 and PGA_1 are interchangeable and thus PGA_1 can be compared with the more characteristic second and final control values. In later stages blood leakage had ceased to be a likely source of error. PGE_1 did not appear to modify the pattern of fluid reabsorption in the upper and middle small intestine. Neither prostaglandin modified the intestinal handling of water in the lower small intestine as far as could be determined. The colon is characterized by its marked ability to reabsorb water. The local administration of PGA_1 ($40 \mu\text{g}$) and PGE_1 ($20 \mu\text{g}$) did not alter this reabsorptive pattern when compared to the second and final control mean values. Paired 't' test of the PGA results in loop B indicated that the difference was not statistically significant ($p > 5\%$). PGE_1 did not appear to induce any change in the intestinal handling of water either in the small bowel or in the colon when administered as a single intraluminal injection of $20 \mu\text{g}$.

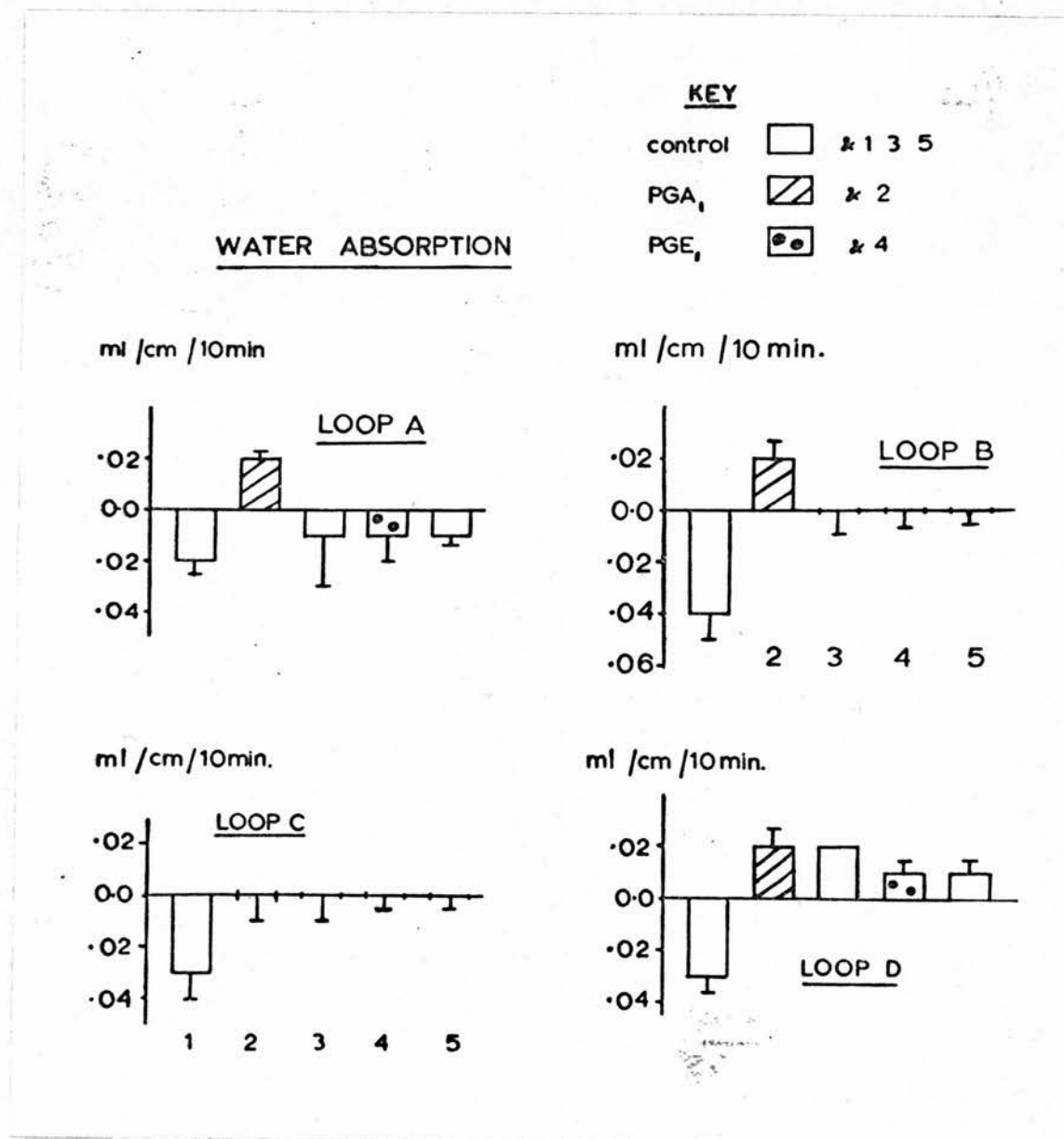
(b) The Effect of Prostaglandins on Net Glucose Flux

The results are summarized in Figure 23b and Table 22b. The initial control values are remarkably high and should not be regarded as valid. The second and final control values are much lower and are more representative of glucose absorption in normal intestine. In the upper and middle and lower small intestine PGE_1 and PGA_1 showed a trend towards depression of glucose absorption.

Figure 23(a)

The Effect of Prostaglandin E₁ and A₁ Infusion on net Water Movement

Values represent the mean of n observations (see Table 22a) \pm S.E.M.



In figures 23a - e the values plotted represent the mean values \pm S.E.M. The positions of PGE₁ and PGA₁ results are interchangeable with respect to the 1st and 2nd and 2nd and 3rd control observations. Details of all of the mean values \pm S.E.M. for these figures are given in tables 22a - e.

Figure 23(b).

The Effect of Prostaglandin E_1 and A_1 on Net Glucose Flux

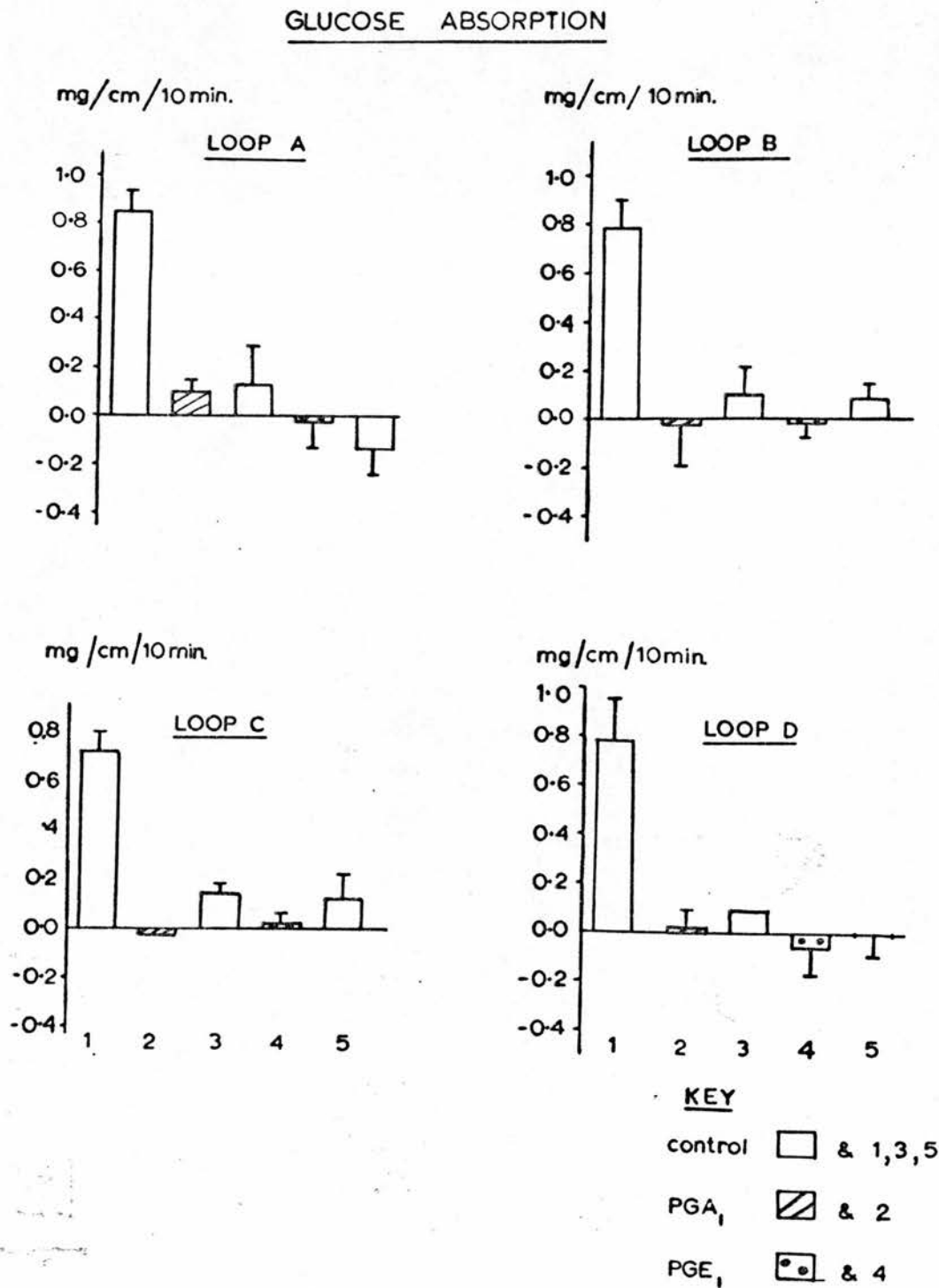


Table 22a. The net flux of water in the piglet gastro-intestinal tract and the effect of local intraluminal injection of PGA_1 (40 μg) and PGE_1 (20 μg) on these fluxes. Loops A-C are situated in the upper, middle and lower small intestine respectively. Loop D is in the spiral colon. The results indicate the mean flux \pm standard deviation of the net water fluxes. The number of observations (n) made for each loop and the standard error of the mean used to construct the graphs, are indicated below the mean values for each loop. The flux was measured as net movement of millilitres of fluid per centimetre length of intestine during the 10 minute observation period.

A. Water - Net Flux

Loop Treatment	A Mean \pm S.D. ml/cm/10min	B Mean \pm S.D. ml/cm/10 min	C Mean \pm S.D. ml/cm/10 min	D Mean \pm S.D. ml/cm/10 min
Control	-0.02 \pm 0.01 n = 9 SEM = 0.005	-0.004 \pm 0.03 n = 9 SEM = 0.01	-0.003 \pm 0.03 n = 7 SEM = 0.01	-0.03 \pm 0.02 n = 8 SEM = 0.006
PGA_1 40 μg	0.02 \pm 0.01 n = 9 SEM = 0.003	0.02 \pm 0.02 n = 8 SEM = 0.008	0.00 \pm 0.02 n = 7 SEM = 0.01	0.02 \pm 0.02 n = 8 SEM = 0.007
Control	-0.01 \pm 0.03 n = 3 SEM = 0.02	0.00 \pm 0.01 n = 3 SEM = 0.008	0.00 \pm 0.02 n = 3 SEM = 0.01	0.02 n = 2 (0.00 & 0.03)
PGE_1 20 μg	-0.01 \pm 0.03 n = 9 SEM = 0.01	0.00 \pm 0.02 n = 9 SEM = 0.006	0.00 \pm 0.01 n = 8 SEM = 0.005	0.01 \pm 0.01 n = 7 SEM = 0.005
Control	-0.01 \pm 0.01 n = 8 SEM = 0.004	0.00 \pm 0.01 n = 7 SEM = 0.004	0.00 \pm 0.01 n = 7 SEM = 0.005	0.00 \pm 0.01 n = 5 SEM = 0.006

Table 22b. The net flux of glucose in piglet small intestine. The Loops A - D are situated in the upper, middle, lower small intestine and spiral colon respectively. The effect of PGA_1 ($40 \mu\text{g}$) and PGE_1 ($20 \mu\text{g}$) on the net glucose flux was investigated. The results given are the means of several observations, indicated below (n), the mean figure, \pm the standard deviation.

B. Glucose Absorption

Loop Treatment	A Mean \pm S.D. mg/cm/10min n SEM	B Mean \pm S.D. mg/cm/10 min n SEM	C Mean \pm S.D. mg/cm/10 min n SEM	D Mean \pm S.D. mg/cm/10 min n SEM
Control	0.84 \pm 0.28 n = 9 SEM = 0.09	0.78 \pm 0.32 n = 9 SEM = 0.08	0.72 \pm 0.22 n = 7 SEM = 0.08	0.78 \pm 0.48 n = 8 SEM = 0.17
PGA_1 40 μg	0.01 \pm 0.15 n = 8 SEM = 0.05	-0.02 \pm 0.48 n = 8 SEM = 0.17	-0.31 \pm 0.23 n = 7 SEM 0.09	0.01 \pm 0.22 n = 8 SEM = 0.08
Control	0.13 \pm 0.20 n = 3 SEM = 0.11	0.10 \pm 0.19 n = 3 SEM = 0.11	0.14 \pm 0.61 n = 3 SEM = 0.04	0.09 n = 2)0.09)0.09
PGE_1 20 μg	-0.03 \pm 0.30 n = 9 SEM = 0.10	-0.01 \pm 0.21 n = 9 SEM = 0.007	0.00 \pm 0.18 n = 8 SEM = 0.063	-0.06 \pm 0.29 n = 7 n = 0.11
Control	-0.07 \pm 0.09 n = 6 SEM = 0.04	0.08 \pm 0.14 n = 6 SEM = 0.06	0.13 \pm 0.22 n = 6 SEM 0.09	0.00 \pm 0.2 n = 5 SEM = 0.09

In these regions of the intestine the mean control value was higher than that of the PGA_1 -treated intestine. The values were, going from upper to lower intestine 0.13 ± 0.20 , 0.10 ± 0.19 , 0.14 ± 0.61 mg/cm/10 minute period for the control tissues and 0.01 ± 0.15 , -0.02 ± 0.48 and -0.31 ± 0.23 in the PGA_1 challenged loops respectively. The differences were not statistically significant ($p > 5\%$). The colon does not absorb glucose normally and prostaglandins did not appear to modify the behaviour of this region of intestine.

(c) The Effect of Prostaglandins on Net Sodium Flux

Prostaglandin-treated and control observations are summarized in Figure 23c and in Table 22c. No marked alteration in the intestinal handling of medium was observed following the administration of prostaglandins in the colonic loop, loop D. In the upper small intestine PGA_1 appeared to enhance sodium absorption. This result was not statistically significantly different from the second control ($p > 5\%$). The mean values in question are 2.60 ± 2.13 mEq/cm/10 minute and -1.97 ± 5.07 mEq/cm/10 minute for PGA_1 and second control respectively. In the middle and lower small intestine both PGA_1 and PGE_1 seemed to increase the mean absorption but again this was not significant ($p > 5\%$). In the colon there was essentially no difference between the prostaglandin sodium net movement figures and the control measurements. The mean values (\pm SD) for the three control readings were 1.75 ± 2.53 , 2.23 (2 observations only), and 1.09 ± 1.85 mEq/cm/10 minutes. PGA_1 sodium value was 1.57 ± 2.3 mEq/cm/10 minutes and that for the corresponding PGE_1 period was 1.45 ± 2.19 mEq/cm/10 minutes.

Figure 23(c).

The Effect of Prostaglandin E_1 and A_1 on Net Sodium Flux

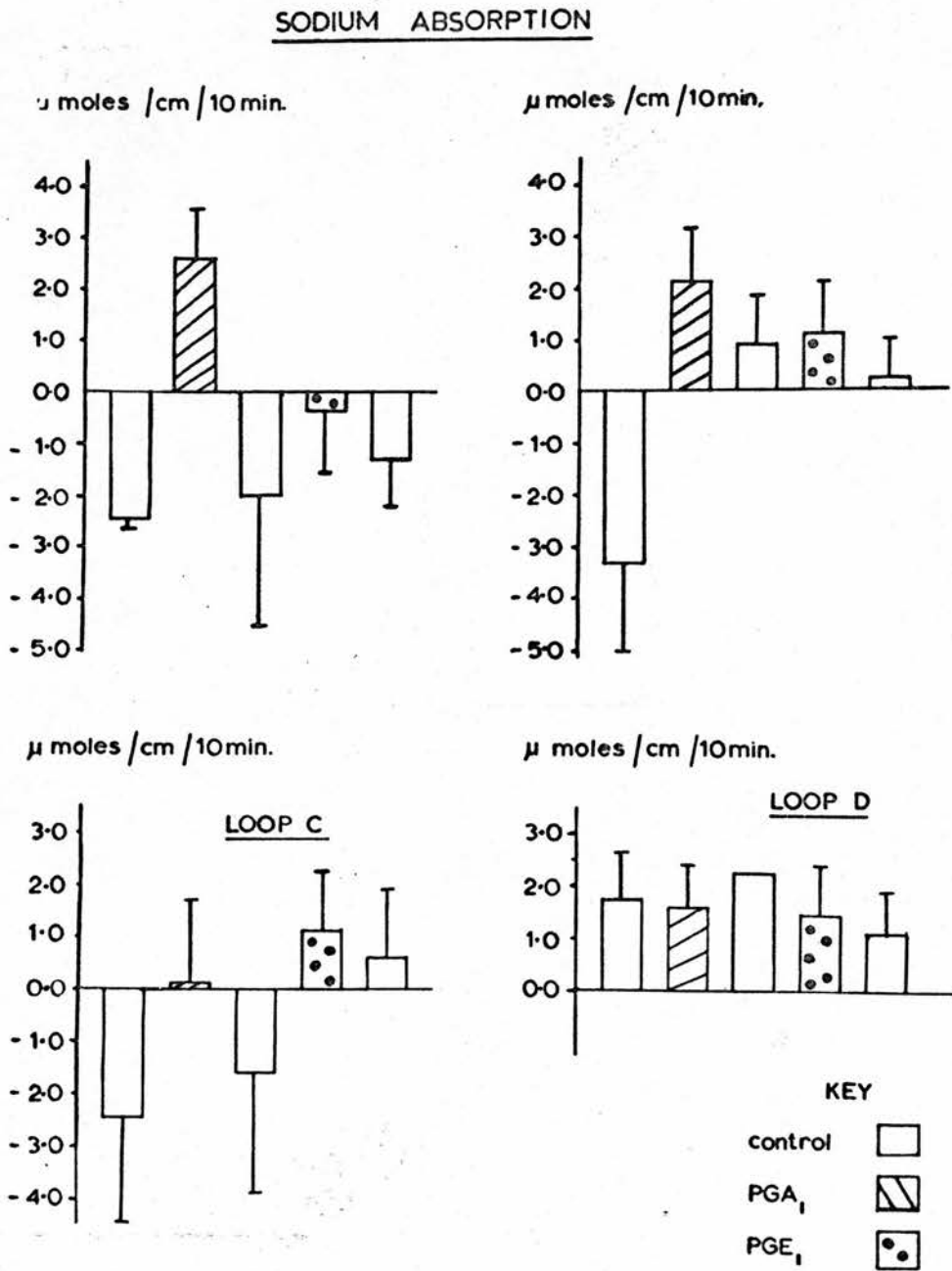


Table 22c. The net sodium flux across piglet small intestine. Loops A - C are situated in the upper, middle and lower small intestine. Loop D is in the spiral colon. The number of observations n, are given below the mean value \pm standard deviation, together with the standard error of the mean.

Net Sodium Flux

Loop Treatment	A $\mu\text{Eq/cm/10 min}$ mean \pm S.D.	B $\mu\text{Eq/cm/10 min}$ mean \pm S.D.	C $\mu\text{Eq/cm/10 min}$ mean \pm S.D.	D $\mu\text{Eq/cm/10 min}$ mean \pm S.D.
Control	-2.48 ± 2.13 n = 9 SEM = 0.17	-3.27 ± 5.14 n = 9 SEM = 1.72	-2.44 ± 5.21 n = 7 SEM = 1.97	1.75 ± 2.53 n = 8 SEM = 0.89
PGA ₁	2.60 ± 2.13 n = 9 SEM = 0.71	2.12 ± 2.9 n = 8 SEM = 1.02	0.10 ± 4.2 n = 7 SEM = 1.59	1.57 ± 2.3 n = 8 SEM = 0.81
Control	-1.97 ± 5.07 n = 3 SEM = 2.93	0.90 ± 2.47 n = 3 SEM = 1.43	-1.6 ± 4.01 n = 3 SEM = 2.03	2.23 n = 2 } 0.00 } 4.46
PGE ₁	-0.33 ± 5.2 n = 9 SEM = 1.7	1.12 ± 2.99 n = 9 SEM = 1.0	1.14 ± 2.4 n = 8 SEM = 0.85	1.45 ± 2.19 n = 7 SEM = 0.83
Control	1.29 ± 2.21 n = 6 SEM = 0.90	0.25 ± 1.84 n = 6 SEM = 0.75	0.59 ± 2.88 n = 5 SEM = 1.29	1.09 ± 1.85 n = 5 SEM = 0.83

Table 22d. The net flux of potassium ions across the small intestine and spiral colon in piglets. Loops A - C are situated in the upper, middle and lower small intestine while Loop D is in the colon. The results are the means of several observations (n) \pm standard deviation. The effect of PGA_1 (40 μg) and PGE_1 (20 μg) on these fluxes was investigated.

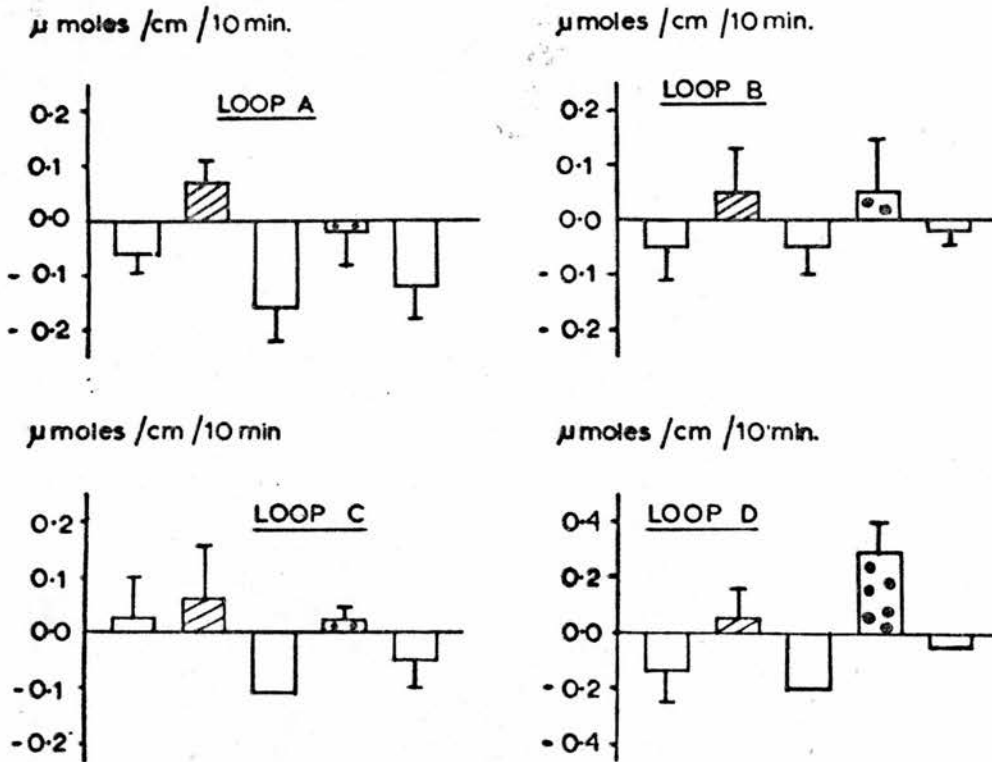
Net Potassium Flux.

Loop Treatment	A $\mu\text{Eq/cm/10min}$ Mean \pm S.D.	B $\mu\text{Eq/cm/10min}$ Mean \pm S.D.	C $\mu\text{Eq/cm/10min}$ Mean \pm S.D.	D $\mu\text{Eq/cm/10min}$ Mean \pm S.D.
Control	-0.06 \pm 0.10 n = 9 SEM = 0.03	-0.05 \pm 0.19 n = 9 SEM = 0.06	0.02 \pm 0.22 n = 7 SEM = 0.08	-0.14 \pm 0.21 n = 4 SEM = 0.11
PGA_1	0.07 \pm 0.13 n = 9 SEM = 0.04	0.05 \pm 0.24 n = 7 SEM = 0.08	0.06 \pm 0.18 n = 7 SEM = 0.07	0.05 \pm 0.16 n = 4 SEM = 0.08
Control	-0.16 \pm 0.06 n = 3 SEM = 0.06	-0.05 \pm 0.09 n = 3 SEM = 0.05	-0.11 n = 2 0.24 0.08	-0.20 n = 2 -0.38 -0.01
PGE_1	-0.02 \pm 0.17 n = 9 SEM = 0.06	0.05 \pm 0.27 n = 9 SEM = 0.09	0.02 \pm 0.07 n = 8 SEM = 0.02	0.29 \pm 0.19 n = 3 SEM = 0.11
Control	-0.12 \pm 0.13 n = 6 SEM = 0.06	-0.02 \pm 0.03 n = 5 SEM = 0.015	-0.05 \pm 0.11 n = 5 SEM = 0.05	-0.05 n = 2 0.32 -0.41

Figure 23 (d)

The Effect of Prostaglandin E₁ and A₁ on Net Potassium Flux

POTASSIUM ABSORPTION



(d) Potassium Ion Movement (net) and prostaglandins

The effect of prostaglandins, PGE_1 and PGA_1 , on the net movement of potassium ions across the small intestine and colon mucosa are depicted in Figure 23d and are contained in Table 22d. As in the case of sodium ions there was no significant alteration in the pattern of potassium ion movement in either the small bowel or in the colon in the presence of PGA_1 and PGE_1 .

(e) The Effect of Prostaglandins on Net Chloride Flux

Neither PGE_1 nor PGA_1 caused significant alteration in the chloride ion flux in the colon. No marked changes occurred in response to PGE_1 (20 μg) administration in the small intestine. With PGA_1 (40 μg), there appeared to be a trend to enhance middle small intestine absorption of chloride ions. The mean PGA_1 stimulated chloride net flux was $2.43 \pm 2.42 \mu\text{Eq/cm/10 minutes}$. The second control net flux for chloride ions was $0.25 \pm 1.12 \mu\text{Eq/cm/10 minutes}$ and the final control was $0.56 \pm 1.79 \mu\text{Eq/cm/10 minutes}$. The difference is not statistically significant ($p > 5\%$). No striking changes were produced by either PGE_1 or PGA_1 in the upper and lower small intestine with the intraluminally injected doses of 40 μg PGA_1 and 20 μg PGE_1 . The results are shown in Table 22e and Figure 23e.

(f) Prostaglandins and Bicarbonate ion Movement

The effect of prostaglandins on bicarbonate ion movement in the small intestine and colon can be seen in Table 22f and Figure 23f. Only in the control and prostaglandin values from this ion does any pattern appear to emerge. The initial control value in all the intestinal loops tends to be depressed by the administration of PGE_1 and PGA_1 . PGA_1 in the lower small intestine seems to cause a more marked trend to secretion of bicarbonate ions than in the upper and middle

Figure 23(e)

The Effect of Prostaglandin E₁ and A₁ on Net Chloride Flux

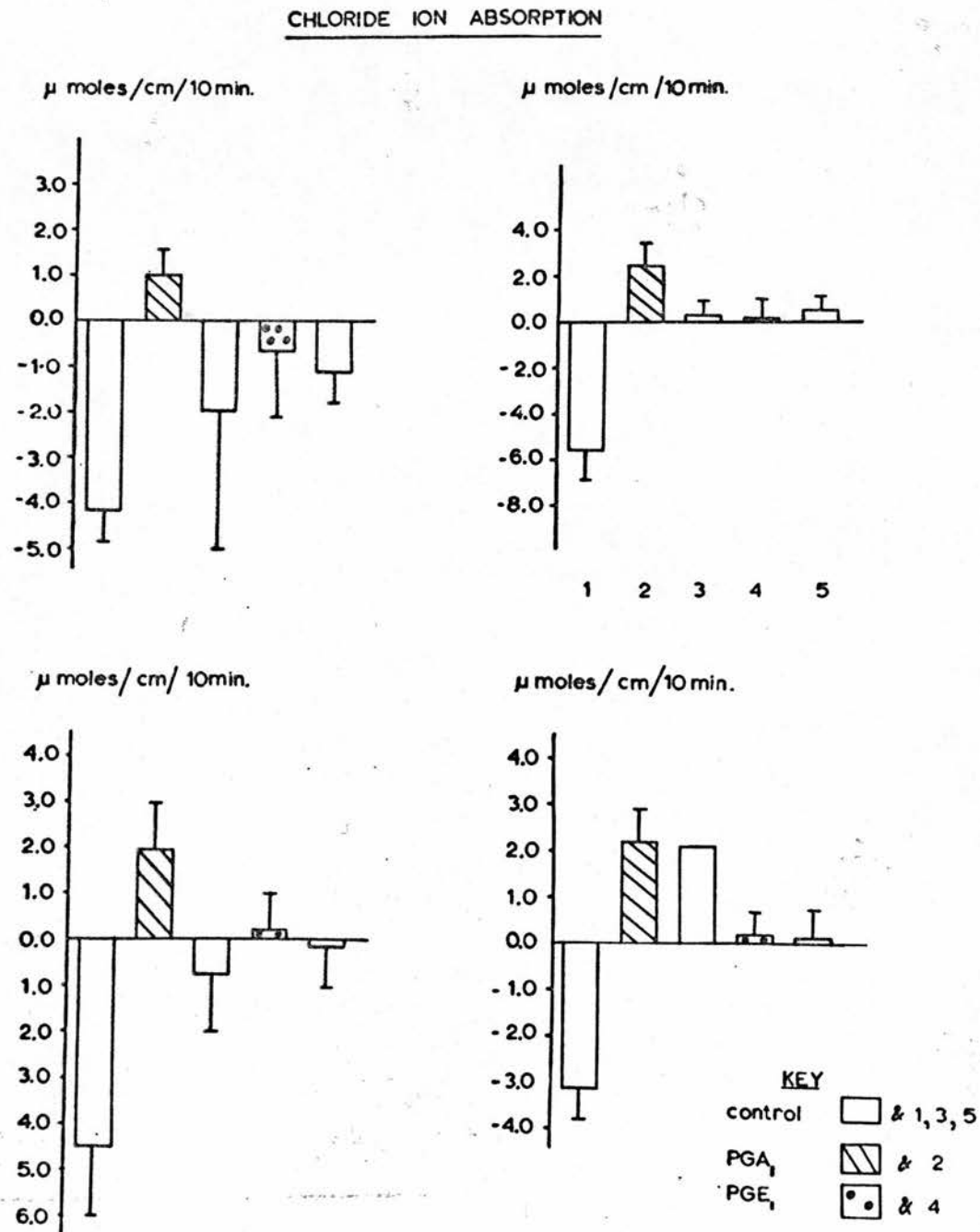


Figure 23(f).

The Effect of Prostaglandin E_1 and A_1 on Net Bicarbonate Flux

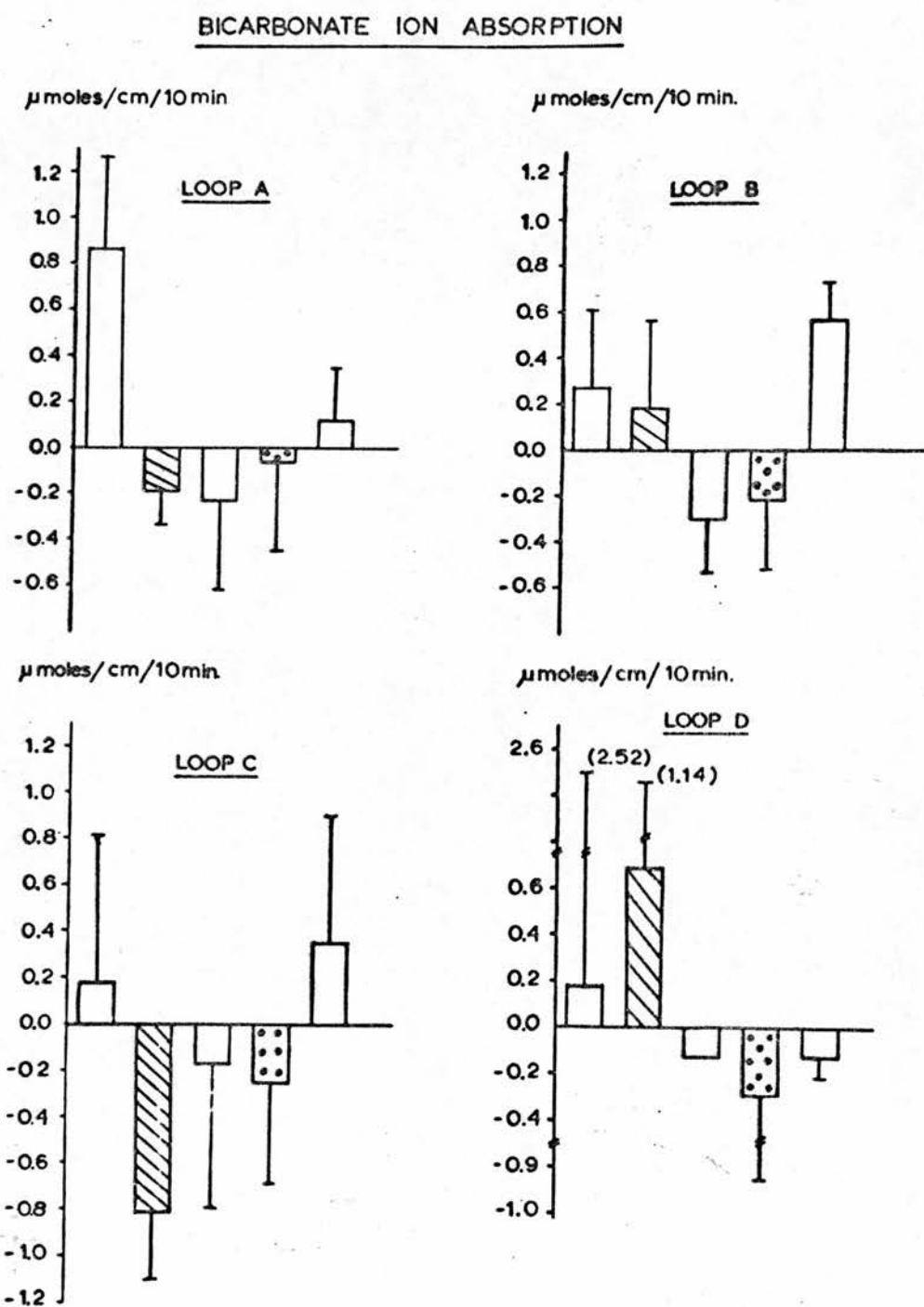


Table 22e. The net flux of chloride ions μEq per centimetre of intestine during the ten minute observation period. Loops A - C are situated in the upper, middle and lower small intestine. Loop D is in the spiral colon. The number of observations (n) made to determine the mean \pm S.D. of each set of values is indicated together with the standard error. The effect of PGA_1 (40 μg) and PGE_1 (20 μg) on these fluxes was determined.

Chloride

Loop Treatment	A $\mu\text{Eq/cm/10 min}$ mean \pm S.D.	B $\mu\text{Eq/cm/10 min}$ mean \pm S.D.	C $\mu\text{Eq/cm/10 min}$ mean \pm S.D.	D $\mu\text{Eq/cm/10 min}$ mean \pm S.D.
Control	-4.34 ± 1.74 n = 9 SEM = 0.58	-5.53 ± 3.65 n = 8 SEM = 1.29	-3.73 ± 3.0 n = 7 SEM = 1.13	-3.15 ± 1.77 n = 7 SEM = 0.67
PGA_1 40 μg	1.06 ± 1.6 n = 9 SEM = 0.54	2.43 ± 2.42 n = 8 SEM = 0.86	0.12 ± 2.54 n = 7 SEM = 0.96	1.71 ± 1.95 n = 8 SEM = 0.69
Control	-1.94 ± 5.10 n = 3 SEM = 2.95	0.25 ± 1.12 n = 3 SEM = 0.65	-0.74 ± 2.15 n = 3 SEM = 1.24	1.59 n = 2 n = 2 } 0.30 } 2.87
PGE_1 20 μg	-0.66 ± 4.48 n = 9 SEM = 1.50	0.19 ± 2.08 n = 8 SEM = 0.74	0.22 ± 2.11 n = 8 SEM = 0.75	0.17 ± 1.15 n = 6 SEM = 0.47
Control	-1.04 ± 1.83 n = 6 SEM = 0.75	0.56 ± 1.79 n = 6 SEM = 0.56	-0.14 ± 2.4 n = 6 SEM = 0.98	0.15 ± 1.24 n = 5 SEM = 0.55

Table 22f. The net flux of bicarbonate ions across the piglet small intestine and the effect of PGA_1 (40 μg) and PGE_1 (20 μg) given by local intraluminal injection on the net ion flux. The results given are the mean values \pm the standard deviations of several observations. The number (n) is indicated below the mean, together with the S.E.M. Loops A - C are situated in the upper, middle and lower small intestine while Loop D is in the spiral colon.

Bicarbonate Ion Flux

Loop	A	B	C	D
Treatment	$\mu\text{Eq/cm/10 min}$ mean \pm S.D.	$\mu\text{Eq/cm/10 min}$ mean \pm S.D.	$\mu\text{Eq/cm/10 min}$ mean \pm S.D.	$\mu\text{Eq/cm/10 min}$ mean \pm S.D.
Control	0.96 ± 0.91 n = 9 SEM = 0.30	0.27 ± 0.95 n = 8 SEM = 0.34	0.17 ± 1.69 n = 7 SEM = 0.64	0.18 ± 3.79 n = 8 SEM = 1.34
PGA_1 40 μg	-0.19 ± 0.48 n = 9 SEM = 0.15	0.18 ± 0.78 n = 8 SEM = 0.28	-0.81 ± 0.89 n = 7 SEM = 0.33	0.64 ± 1.58 n = 8 SEM = 0.53
Control	-0.22 ± 0.70 n = 3 SEM = 0.40	-0.30 ± 0.40 n = 3 SEM = 0.23	-0.17 ± 1.16 n = 3 SEM = 0.67	-0.15 n = 2 n = 2 } 0.04 } -0.33
PGE_1 20 μg	-0.06 ± 1.16 n = 9 SEM = 0.39	-0.23 ± 0.82 n = 8 SEM = 0.29	-0.25 ± 1.22 n = 8 SEM = 0.43	-0.28 ± 1.77 n = 7 SEM = 0.67
Control	0.12 ± 0.56 n = 6 SEM = 0.23	0.56 ± 0.39 n = 6 SEM = 0.16	0.35 ± 1.5 n = 6 SEM = 0.61	-0.12 ± 0.20 n = 5 SEM = 0.09

small intestine. The second control value shows a net secretion at all points along the small intestine. In the upper small intestine 0.2 ± 0.7 μEq bicarbonate per cm per 10 minutes is secreted. The corresponding values for the middle and lower small intestine are 0.3 ± 0.4 μEq and 0.17 ± 1.16 μEq per cm per 10 minute observation period. The third and final control value shows a return to absorption of bicarbonate ions. The mean absorption of bicarbonate ions was 0.12 ± 0.56 , 0.56 ± 0.39 and 0.35 ± 1.5 μEq bicarbonate per centimetre length of intestine during the ten minute study period. The middle and lower small intestine appear to be more sensitive to the effects of PGE_1 than does the upper small intestine but the range of values encountered do preclude any definite statement on this possibility.

No significant alteration in net bicarbonate ion movement was observed in the colon.

Discussion

It was anticipated that with the high doses of prostaglandin used and even allowing for substantial metabolism of these compounds by the intestinal mucosa, ~~that~~ some marked changes in net ion flux across the intestinal mucosa would be observed. It would seem that the piglet intestinal mucosa is either capable of metabolizing the prostaglandin presented to it from the luminal side of the mucosal cell very rapidly and effectively, or possibly is particularly insensitive to the effects of intraluminally administered prostaglandins. The only ion flux which may be considerably altered by prostaglandins appears to be the bicarbonate ion flux. (The significance of these findings in relation to the actions of *E. coli* toxin on ion and water movement will be discussed together with the remainder of the results in this section and in conjunction with the

calf perfusion results).

Bywater and Jeffries (unpublished observations) have investigated the effects of heat-stable *Escherichia coli* toxin preparations on water, glucose and electrolyte movement in the small intestine of healthy neonatal piglets both in vivo and in vitro. Using the everted intestinal sac model it has been observed that the uptake of glucose by the intestinal mucosa is markedly depressed by exposure of the tissue to *E. coli* heat-stable toxin. Secondary to this effect, is the reduction in water absorption by the mucosa. In vivo, preliminary investigations using a perfused intestinal loop model have confirmed these results. The net movement of sodium, potassium, chloride and bicarbonate ions was also studied in this latter model. Neither potassium, nor chloride nor bicarbonate ion fluxes showed any alteration from their usual pattern following toxin administration. The absorption of sodium was slightly depressed but this was not statistically significant and was probably a reflection of the depression of the glucose-dependent sodium transport pathway which operates in the gastro-intestinal mucosal cells (Bywater and Jeffries, unpublished observations).

Thus it is evident that prostaglandins do not imitate the effects of heat-stable *E. coli* toxin in the small intestine. PGE_1 and PGA_1 did not produce a significant reduction in glucose absorption, in the doses used, but did promote bicarbonate ion secretion; the toxin depressed glucose uptake but caused no alteration in the net bicarbonate ion movement across the mucosa of the small intestine.

Conclusion

Prostaglandins stimulate bicarbonate ion secretion in piglet small intestine. Neither PGA_1 nor PGE_1 in the doses used showed any modification of intestinal handling of water, glucose, sodium or potassium or chloride. It was therefore concluded that prostaglandins of the E and A series are unlikely to be mediators of the secretory activity stimulated by *Escherichia coli* toxin in the small intestine.

Experiment 5

The intra-arterial infusion of prostaglandins in calves

The effect of *Escherichia coli* heat stable toxin on ion and water fluxes in the small intestine and colon of calves has been extensively investigated by Bywater and Jefferies (in preparation). It was possible that prostaglandins might be able to simulate the effects of *E. coli* toxin on ion movement in the small intestine and colon. Pierce and his associates demonstrated that prostaglandins administered intra-arterially in dogs are capable of stimulating fluid secretion in the small bowel (Pierce et al, 1971). Cholera toxin stimulates copious fluid production in the calf intestine (Bywater, unpublished observations).

Methods

Four calves weighing 30-40 kg and 5-10 days old, were anaesthetised with pentobarbitone sodium 25 mg/kg injected intravenously. The trachea was intubated and the animals were allowed to breathe oxygen/carbon dioxide (95:5 per cent). The abdomen was opened and lengths of intestine at approximately 15 p. 100, 40 p 100, 65 p 100, and 90 p 100 of the distance from the pylorus to the ileo-caecal valve were selected. The positions were confirmed at the end of the experiment. A 15 cm length of intestine was tied off with silk ligatures. The loops were cannulated at their upper ends with a multiperforate 15 cm long cannula which was passed through the centre of a nylon bobbin. The bobbin was then secured with a silk ligature at the upper end of the loop. Animals received a continuous intravenous infusion of isotonic saline at a rate of 10 ml/min. via the jugular vein.

A fine cannula was inserted into one of the smaller branches

of the superior mesenteric artery close to the intestinal wall, in the gut mesentery, and was passed retrogradely until the tip lay just below the point at which the superior mesenteric artery branched from the aorta. The position of this cannula was confirmed at death. During control observation periods 0.9% saline was infused into the superior mesenteric artery at a rate of 1-1.5 ml per minute. During test, prostaglandin observations, prostaglandin dissolved in 0.9% saline were infused via the arterial cannulae at a rate of 1-1.5 ml per minute. The rate was maintained constant using a Braun infusion pump system for both the control and test infusions.

The lumen of each loop was washed with warm saline and a 15 ml of an isotonic test solution was injected into each of the loops to be studied. The test solution contained sodium chloride (4.772 g/l), sodium bicarbonate (2.185 g/l), potassium chloride (0.2985 g/l) glucose (10g/l) and either polyethylene glycol 4000 (7.5 g/l) or phenol red (50 µg/ml). ^{22}Na (2 µCi/l) and D_2O p. 100 w/v were added to the solution. The solution was mixed rapidly with the residual saline in the loop and an initial sample of 0.2 ml was removed for duplicate estimations of polyethylene glycol or phenol red. This procedure was adopted in order to determine the amount of residual saline in the intestinal loops. After a fifteen minute period the loops were drained and the control sample was taken.

Prostaglandin administration

(1) $\text{PGF}_{2\alpha}$ $\text{PGF}_{2\alpha}$ 2 µg/ml - 25 µg/ml was infused at a rate of 1-1.5 ml per minute for 50 minutes in 3 calves into the superior mesenteric artery. Measurements of the ion fluxes and water movement were taken in the last 15 minutes of the perfusion period.

(2) PGE_2 . PGE_2 2 $\mu\text{g}/\text{ml}$ - 50 $\mu\text{g}/\text{ml}$ was infused at a rate of 1 ml/minute for 50 minutes in 4 calves via the superior mesenteric artery. As in the case of $\text{PGF}_{2\alpha}$ ion and water fluxes were estimated during the final 15 minutes of the infusion period.

(3) PGA_1 . PGA_1 2 μg - 25 $\mu\text{g}/\text{ml}$ was infused at rates of 1 to 1.5 ml per minute for 40 or 50 minute periods via the superior mesenteric artery in 3 animals and any changes in ion flux and water absorption were measured during the last 15 minutes of perfusion.

A 60 - 90 minute control period was observed between each prostaglandin infusion. The last 50 minutes of this control period was used to infuse 0.9% for the same length of time as the prostaglandin solution and to record control, non-prostaglandin-treated intestinal loop ion and water movement in the loops. Again a 15 minute observation period was employed.

Ion and Water Flux Determinations

The methods used to determine potassium, sodium chloride and bicarbonate ion fluxes are described in Experiment 4, Section 2. The method used to estimate water movement is also described in Experiment 4 for phenol red containing samples. In the experiment in which polyethylene glycol was used to estimate the volume and correct the ion concentrations, the polyethylene glycol was estimated by the turbidometric method of Hyden (1956) and modified by Smith (1959) and by Bywater (1973b). Deuterium oxide was measured as described by Bywater (1973b). The net ion and water movements were estimated and used to compare the effects of prostaglandin infusion with *Escherichia coli* toxin results (obtained by Bywater and Jefferies).

Results

The effects of infusing prostaglandins into the superior

mesenteric artery for 40 to 50 minutes in doses of 2-50 $\mu\text{g/ml}$ at rates of 1-1.5 ml per minute are summarized in Tables 23-25. The results are also shown in histogram form in Figures 24 to 26. Only net fluxes were investigated.

(a) PGF_{2 α} infusion

Intra-arterial infusion of PGF_{2 α} in doses of 2-25 $\mu\text{g/ml/minute}$ did not appear to induce any significant alteration in the ion and water flux across the mucosa of the small intestine in calves.

(b) PGA₁ infusion

The administration of PGA₁ did not significantly alter the net flux of water across the small intestine at any level. The flux of sodium ions in the upper and middle small bowel did not appear to be modified in any way. In the lower small intestine there appeared to be a depression of sodium absorption but the difference between PGA₁ treatment and control observations was not statistically significant. The effects were measured in 3 animals only. Potassium flux was not modified by PGA₁ treatment nor were the chloride or bicarbonate net fluxes.

(c) PGE₂ infusion

As in the experiments in which PGA₁ and PGF_{2 α} were infused, little alteration in net ion and water fluxes were elicited by PGE₂. No enhanced or diminished net movement of potassium, water, sodium or chloride was encountered. Bicarbonate ion flux was however altered. Three of the four loops A (15% duodenum-ileocaecal valve distance), C (65% duodenum-ileocaecal valve interval), and D (90% of duodenum-ileocaecal valve distance) were observed to show an enhanced bicarbonate secretion. The mean output of bicarbonate ions in control observation periods by these loops was $15.69 \pm 15.71 \mu\text{Eq/15 minutes/100 cm}^2$, $-4.77 \pm 18.43 \mu\text{Eq/15 minutes/100 cm}^2$ and

Calif. Superior Mesenteric Artery Perfusion Experiment

Table 23. The effect of intra-arterial PGE₂ infusion on net ion and water fluxes in the small intestine of calves

PGE₂ perfusion

Net H₂O Flux ml / 100cm² / 15min

c	Loop A	B	C	D
Mean \pm s.d. con	2.059 \pm 3.71	2.45 \pm 3.24	1.64 \pm 2.36	2.85 \pm 1.77
Mean \pm s.d. P.G.	4.55 \pm 8.26	2.21 \pm 6.02	-1.74 \pm 7.75	2.64 \pm 1.50
S.E.M.	1.17; 3.37	2.46; 1.03	2.69; 5.57	0.61; 0.56
t	N.S. *	N.S.	N.S.	N.S.

Net K⁺ Flux μ moles / 100cm² / 15min

con	37.5 \pm 16.82	-37.3 \pm 14.7	-42.7 \pm 22.92	-2.46 \pm 15.46
P.GE ₂	37.8 \pm 11.59	-39.85 \pm 10.39	-41.99 \pm 48.00	0.39 \pm 8.08
S.E.M.	5.32; 4.73	4.90; 4.24	9.36; 15.20	4.89; 3.03
t	N.S.	N.S.	N.S.	N.S.

Net Na Flux μ moles / 100cm² / 15min

con	-176.6 \pm 486.5	-225.92 \pm 449.11	-75.02 \pm 315.73	154.0 \pm 371.5
P.GE ₂	564.56 \pm 1004.0	-203.1 \pm 654	399.58 \pm 1312.6	113.9 \pm 419.56
S.E.M.	409.9; 153.84	267.32; 142.02	535.72; 99.84	117.50 \pm 171.29
t	N.S.	N.S.	N.S.	N.S.

Net Chloride Flux μ moles / 100cm² / 15min

con	-614.41 \pm 457.98	-556.61 \pm 360.98	-147.99 \pm 261.12	271.59 \pm 288.26
P.GE ₂	-60.23 \pm 1204.1	-370.08 \pm 825	-488.05 \pm 701.83	235.45 \pm 260.86
S.E.M.	144.83; 491.59	114.15; 336.82	82.57; 286.53	91.16; 106.5
t	N.S.	N.S.	N.S.	N.S.

Net Bicarbonate Flux μ moles / 100cm² / 15min

con	15.69 \pm 15.71	9.58 \pm 28.4	-4.77 \pm 18.43	-5.39 \pm 9.57
P.GE ₂	-0.13 \pm 11.65	-5.22 \pm 16.11	-34.87 \pm 26.55	-15.58 \pm 6.0
S.E.M.	4.80; 4.76	8.91; 6.58	5.83; 10.84	3.03; 2.45
t	5%	N.S.	5%	5%

* N.S. = not statistically significant at the 5% level.

Table 24. The effect of intra-arterial infusion of PGF_2 on net ion and water fluxes in the calf small intestine

The effect of PGF_2 infusion on Net Ion Fluxes.

Net H_2O Flux ($\text{ml} / 100 \text{ cm}^2 / 15 \text{ min}$)

con.	2.38 ± 5.19	2.57 ± 3.22	0.78 ± 4.98	2.73 ± 3.5
$\text{P.G.F}_{2\alpha}$	-1.28 ± 4.01	5.26 ± 3.48	4.92 ± 3.66	3.6 ± 0.78
S.E.M.	2.11; 2.32	1.32; 2.01	2.03; 2.11	1.43; 0.45
t, significance	N.S.	N.S.	N.S.	N.S.

Net Na Flux ($\mu\text{moles} / 100 \text{ cm}^2 / 15 \text{ min}$)

con	-24.85 ± 544.34	-127.52 ± 338.52	327.03 ± 631.39	355.5 ± 463.0
$\text{P.G.F}_{2\alpha}$	-649.53 ± 452.62	90.73 ± 491.31	558.1 ± 883.58	264.9 ± 338.39
S.E.M.	222.23; 261.3	138.21; 283.67	257.77; 510.15	189.03; 195.37
t	N.S.	N.S.	N.S.	N.S.

Net K Flux ($\mu\text{moles} / 100 \text{ cm}^2 / 15 \text{ min}$)

con	-46.2 ± 23.89	-45.4 ± 24.91	-42.33 ± 45.6	-5.12 ± 14.2
$\text{P.G.F}_{2\alpha}$	-49.63 ± 32.4	-29.83 ± 45.4	-36.03 ± 23.35	-2.7 ± 3.83
S.E.M.	9.76; 18.71	10.16; 26.23	18.62; 13.48	5.80; 2.21
t	N.S.	N.S.	N.S.	N.S.

Net Cl. Flux ($\mu\text{moles} / 100 \text{ cm}^2 / 15 \text{ min}$)

con	-881.68 ± 677	-688.23 ± 276	-263.6 ± 507	-340.88 ± 450.43
$\text{P.G.F}_{2\alpha}$	-1014.83 ± 380	-396.26 ± 246.96	-318 ± 673.4	-484.03 ± 214.38
S.E.M.	276.69; 219.72	1112.8; 142.58	207.0; 388.3	183.90; 123.78
t	N.S.	N.S.	N.S.	N.S.

Net HCO_3 Flux ($\mu\text{moles} / 100 \text{ cm}^2 / 15 \text{ min}$)

con	16.72 ± 26.87	6.05 ± 37	-4.4 ± 22	-8.32 ± 5.85
$\text{P.G.F}_{2\alpha}$	23.17 ± 6.14	36.64 ± 17.24	-10.13 ± 24.66	-18.1 ± 8.47
S.E.M.	10.97; 3.55	5.46; 9.95	9.05; 14.24	2.39; 4.89
t	N.S.	N.S.	N.S.	N.S.

Table 25. The effect of intra-arterial infusion of PGA_1 on net ion and water fluxes in the calf small intestine

The Effect of PGA_1 infusion on Net Ion Fluxes

(a) H_2O Flux ($\text{ml}/100\text{cm}^2/15\text{min}$)

Con.	2.34 ± 2.29	-0.95 ± 3.5	0.04 ± 4.72	2.99 ± 1.29
PGA_1	0.94 ± 3.44	2.19 ± 3.07	-0.59 ± 3.99	1.60 ± 1.50
S.E.M.	0.93; 1.98	1.4; 1.77	1.93; 2.31	0.53; 0.86
t	N.S.	N.S.	N.S.	N.S.

(b) Na Flux ($\mu\text{moles}/100\text{cm}^2/15\text{min}$)

Con	-84.62 ± 271.36	779.93 ± 360.59	-397.52 ± 604.17	299.63 ± 144.23
PGA_1	107.83 ± 171.7	-219.6 ± 457.1	-415.3 ± 568.95	94.63 ± 109.91
S.E.M.	110.78; 99.13	147.22; 263.92	246.66; 328.49	58.88; 63.46
t	N.S.	N.S.	N.S.	N.S.

(c) K Flux ($\mu\text{moles}/100\text{cm}^2/15\text{min}$)

Con	-32.95 ± 13.44	-48.22 ± 18.47	-42.32 ± 42.17	-4.7 ± 14.1
PGA_1	1.43 ± 51.27	-20.80 ± 24.65	-22.5 ± 9.02	12.37 ± 8.06
S.E.M.	5.49; 29.61	7.54; 14.23	17.22; 5.21	5.76; 4.65
t	N.S.	N.S.	N.S.	N.S.

(d) Cl. Flux ($\mu\text{moles}/100\text{cm}^2/15\text{min}$)

Con	-575.87 ± 255.25	-526.87 ± 857.13	-293.0 ± 448.8	422.12 ± 225.35
PGA_1	-481.77 ± 214.68	-652.67 ± 46.25	-315.57 ± 480.04	190.6 ± 154.92
S.E.M.	104.21; 123.95	349.94; 26.70	183.23; 277.16	92.0; 89.45
t	N.S.	N.S.	N.S.	N.S.

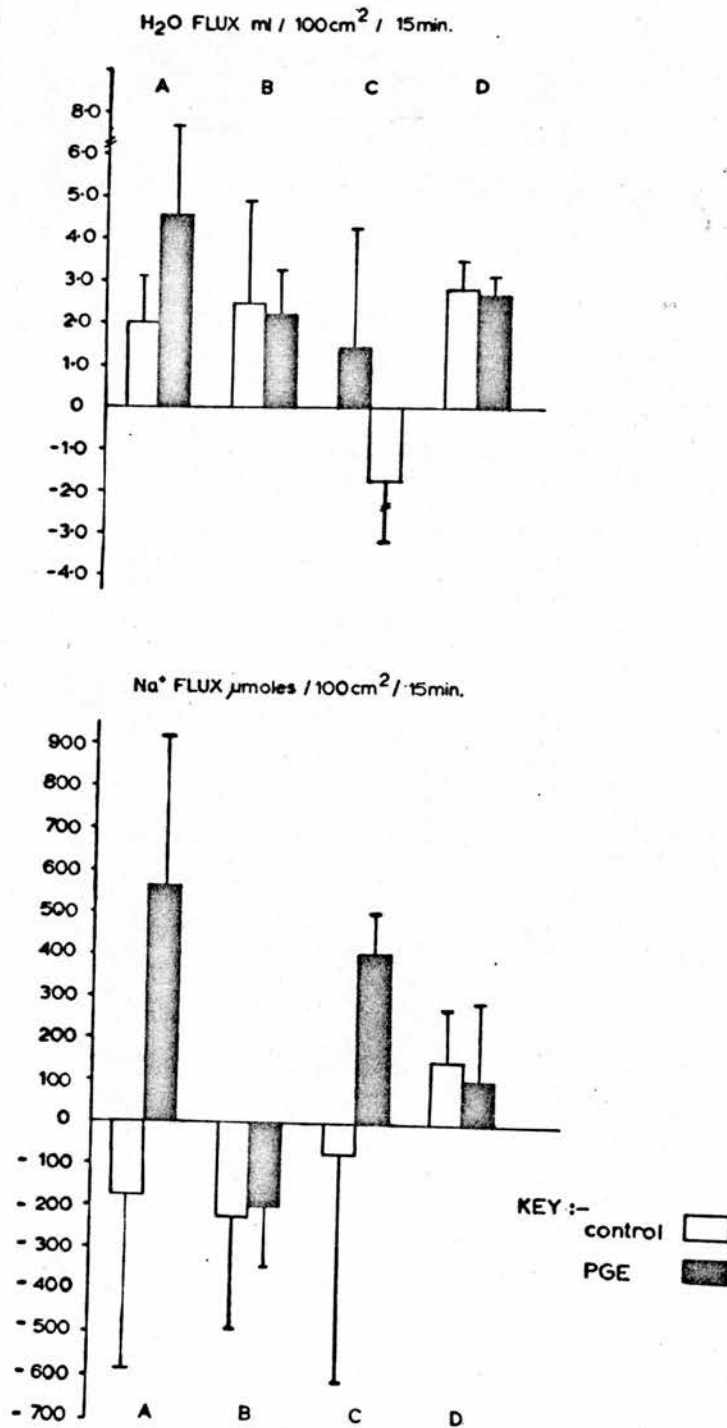
(e) HCO_3 Flux ($\mu\text{moles}/100\text{cm}^2/15\text{min}$)

Con	24.42 ± 11.42	21.65 ± 13.71	-15.85 ± 21.55	-7.57 ± 9.53
PGA_1	22.37 ± 19.38	17.87 ± 5.01	-21.17 ± 28.84	-12.6 ± 8.64
S.E.M.	4.66; 11.19	5.60; 2.89	8.80; 16.65	3.89; 4.99
t	N.S.	N.S.	N.S.	N.S.

Figure 24

The Effect of PGE₂ Infusion on Water and Sodium Flux

THE EFFECT OF PGE INFUSION ON NET WATER AND ION FLUXES



The values plotted represent the mean change in net flux of n observations \pm S.E.M. (Table 23).

Figure 24 - contd.

The Effect of PGE₂ Infusion on Net Potassium Chloride and Bicarbonate Flux

THE EFFECT OF PGE INFUSION ON NET ION FLUXES

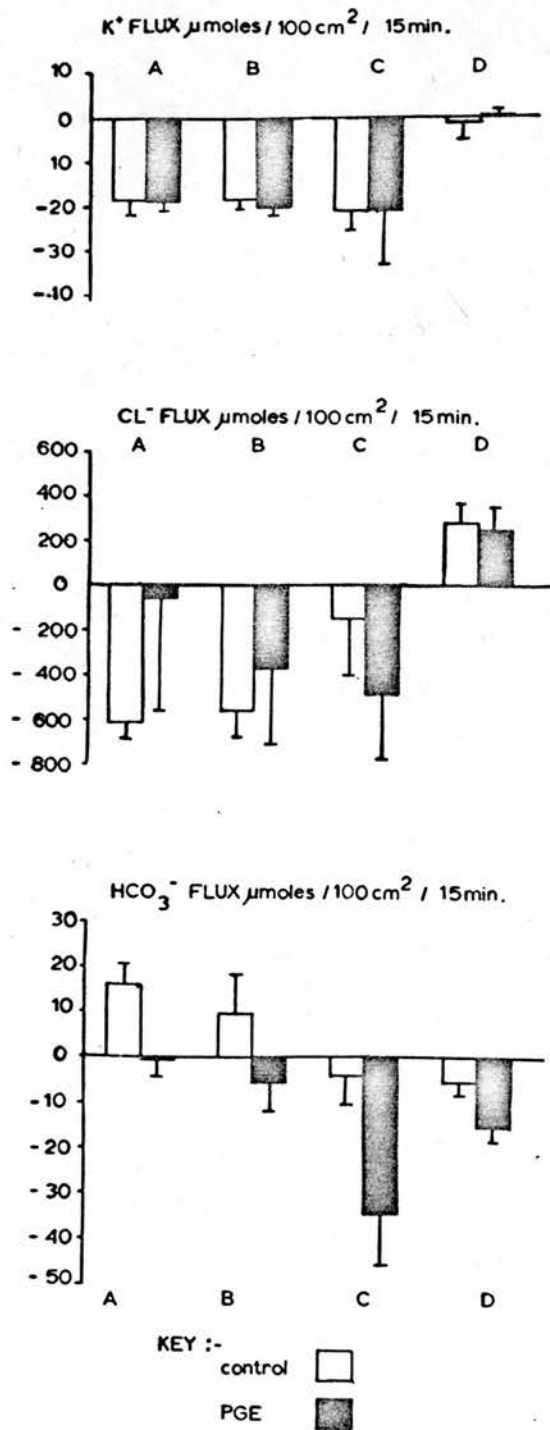
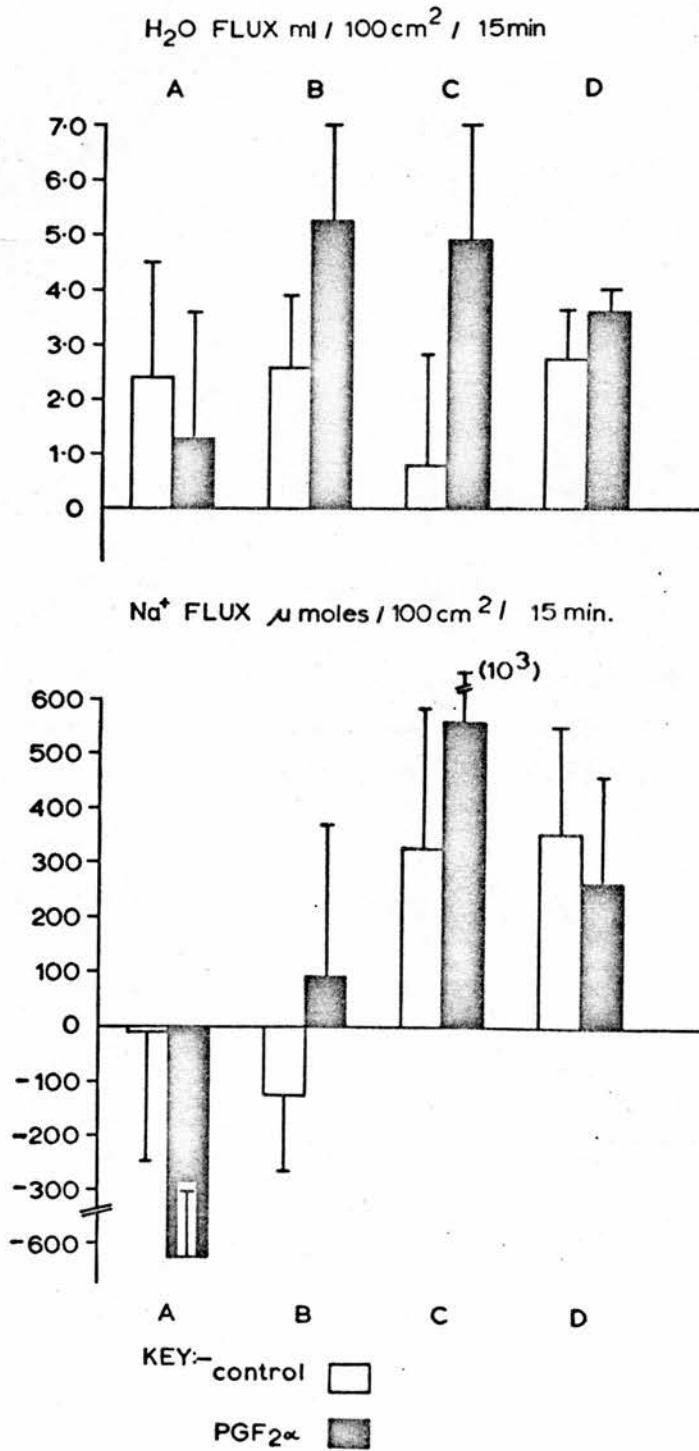


Figure 25

The Effect of PGF_2 Infusion on Net Water and Sodium Flux

THE EFFECT OF $\text{PGF}_2\alpha$ INFUSION ON WATER AND NET ION FLUXES



The values shown represent the mean change in net flux of n observations \pm S.E.M. - see Table 24 for details.

Figure 25 - contd.

The Effect of PGF_2 Infusion on Net Potassium Chloride and Bicarbonate Flux

THE EFFECT OF PGF_2 INFUSION ON NET ION FLUXES

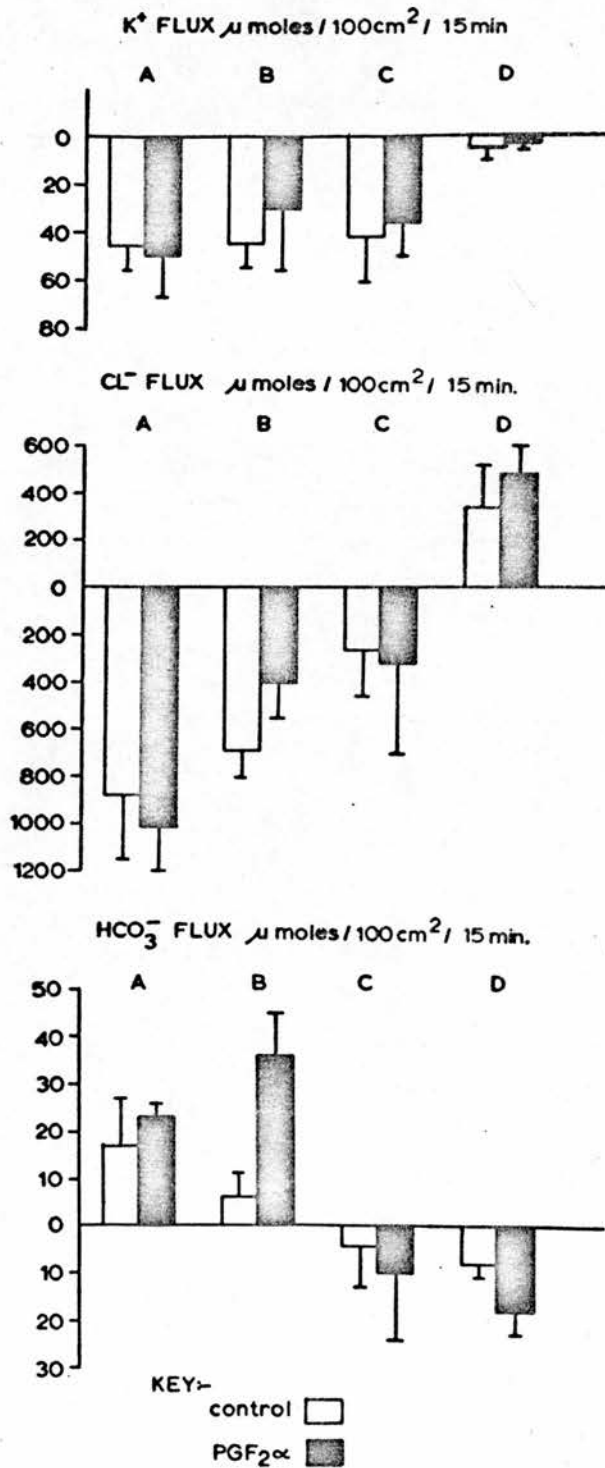
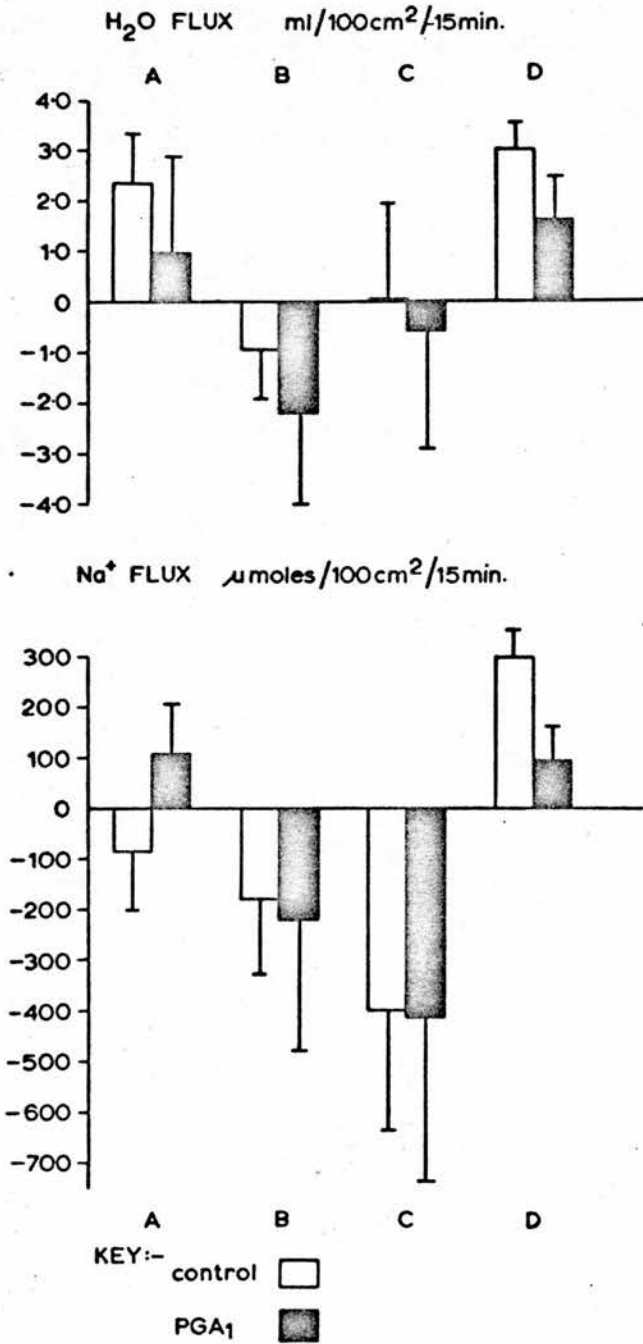


Figure 26

The Effect of PGA_1 Infusion on Net Water and Sodium Flux

THE EFFECT OF PGA_1 INFUSION ON WATER AND NET ION FLUXES

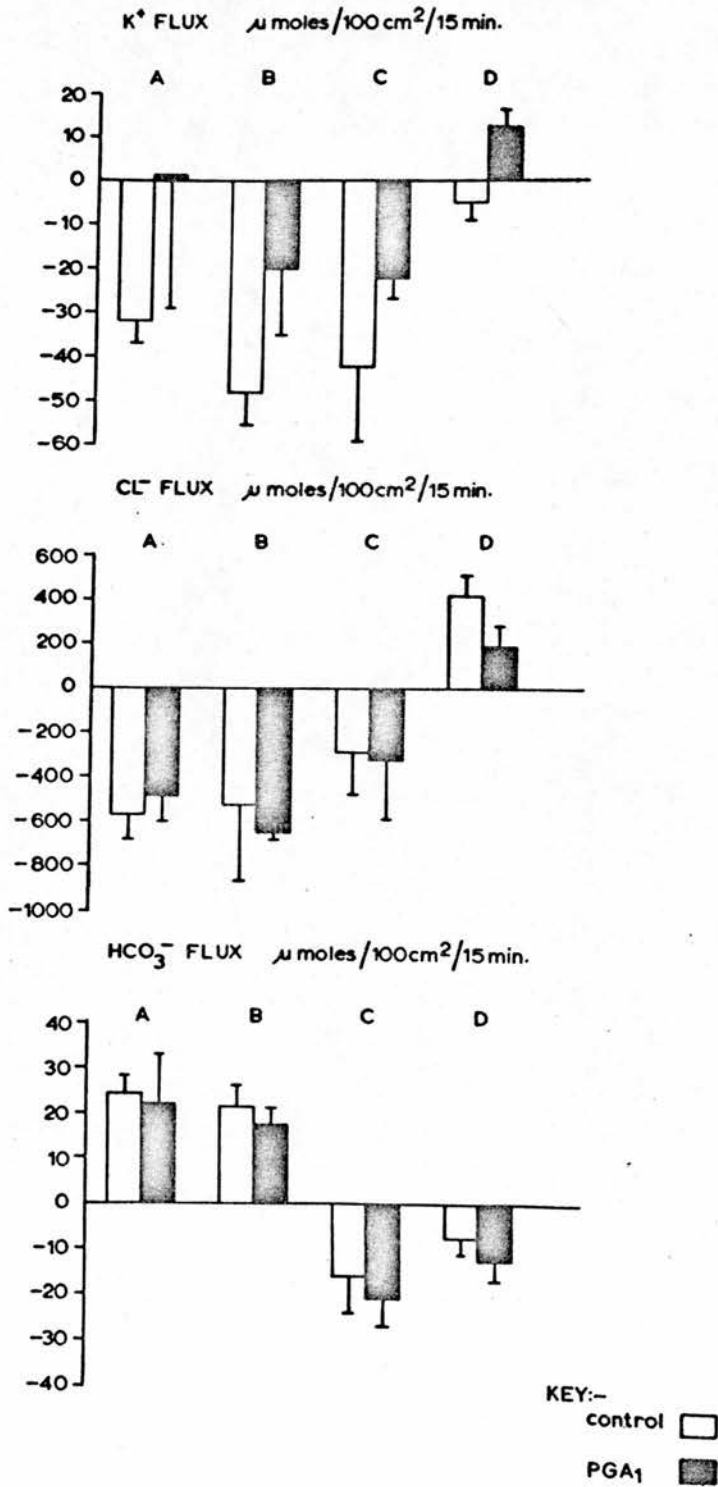


The values shown represent the mean change in net flux of n observations \pm S.E.M. - see Table 25 for details.

Figure 26 - contd.

The Effect of PGA_1 Infusion on Net Potassium Chloride and Bicarbonate Flux.

THE EFFECT OF PGA_1 INFUSION ON NET ION FLUXES



$-5.39 \pm 9.57 \mu\text{Eq}/15 \text{ minutes}/100 \text{ cm}^2$ respectively. The corresponding values after prostaglandin E_2 administration were -0.13 ± 11.65 , -34.87 ± 26.55 and $-15.58 \pm 6.0 \mu\text{Eq}/15 \text{ minutes}/100 \text{ cm}^2$. The fourth loop, loop B, at 40% of the distance between the duodenum and the ileocaecal valve did show a similar pattern of response to PGE_2 but the results obtained in control and prostaglandin-treated intestinal loops were not statistically significantly different ($p > 5\%$). The values were $9.58 \pm 28.4 \mu\text{Eq}$ and $-5.22 \pm 16.11 \mu\text{Eq}/15 \text{ minutes}/100 \text{ cm}^2$ in the untreated and prostaglandin treatment segments of intestine.

Discussion

Neither PGA_1 (2-25 $\mu\text{g}/\text{ml}$ infused at a rate of 1 ml/minute for 50 minutes) nor $\text{PGF}_{2\alpha}$ (2-25 $\mu\text{g}/\text{ml}$ infused for 50 minutes at a rate of 1 ml/minute) were found to modify the intestinal handling of water and electrolytes. PGE_2 alone (2-50 $\mu\text{g}/\text{ml}$ for 50 minutes at an infusion rate of 1.15 ml/minute) when given via the superior mesenteric artery was observed to alter net ion flux across the intestinal mucosa. The ion affected was the bicarbonate ion. PGE_2 in the doses used enhanced the exsorption or secretion of this particular ion.

These results with prostaglandins contrast sharply with those obtained by Bywater and Jeffries who, using a similar calf model, studied the effects of heat stable *Escherichia coli* toxin on the movement of ions and water across the intestinal mucosa. In these investigations the toxin depressed water absorption in the upper small intestine and stimulated net secretion of fluid in the lower half of the small bowel. Sodium ions were secreted in the upper intestine in healthy calves and this output was not augmented

by toxin administration. In the lower gut, however, sodium ions were normally absorbed and this movement was reversed by heat-stable toxin. The secretion of potassium ions was enhanced by challenging the intestine with *E. coli* toxin but this response was only elicitable in the terminal ileum. The pattern of chloride ion movement followed that of sodium ions in toxin treated intestine. Thus net secretion was found in the lower small bowel in infected animals but not in healthy control calves. The net movement of bicarbonate ions was not sensitive to *E. coli* heat-stable toxin. All of these responses differed from those observed following prostaglandin infusion. These results are shown in Figures 27 to 31 which were provided by Dr. Bywater of the Royal (Dick) School of Veterinary Studies (Edinburgh).

Conclusion

PGE_2 (2-50 $\mu\text{g/ml}$) administered by intra-arterial infusion via the superior mesenteric artery stimulated bicarbonate ion secretion in the small intestine in calves. PGA_1 (2-25 $\mu\text{g/ml}$) and $\text{PGF}_{2\alpha}$ (2-25 $\mu\text{g/ml}$) did not modify bicarbonate ion movement in the small bowel. Neither PGE_2 , $\text{PGF}_{2\alpha}$ nor PGA_1 influence the net fluxes of water, sodium, potassium or chloride ions across the intestinal mucosa in healthy calves. It was therefore concluded that prostaglandins were unlikely to be the mediators of *E. coli* heat-stable toxin-induced fluid and electrolyte secretion in the small bowel.

Figure 27. The effect of *E. coli* toxin on net water movement in calf intestine. Values represent mean change \pm S.E.M. The statistical significance is indicated where appropriate in this and subsequent figures (Figures 28 - 31). The position of the loops in the small intestine is indicated on the horizontal axis as percentage distance from the duodenum, towards the ileocaecal valve.

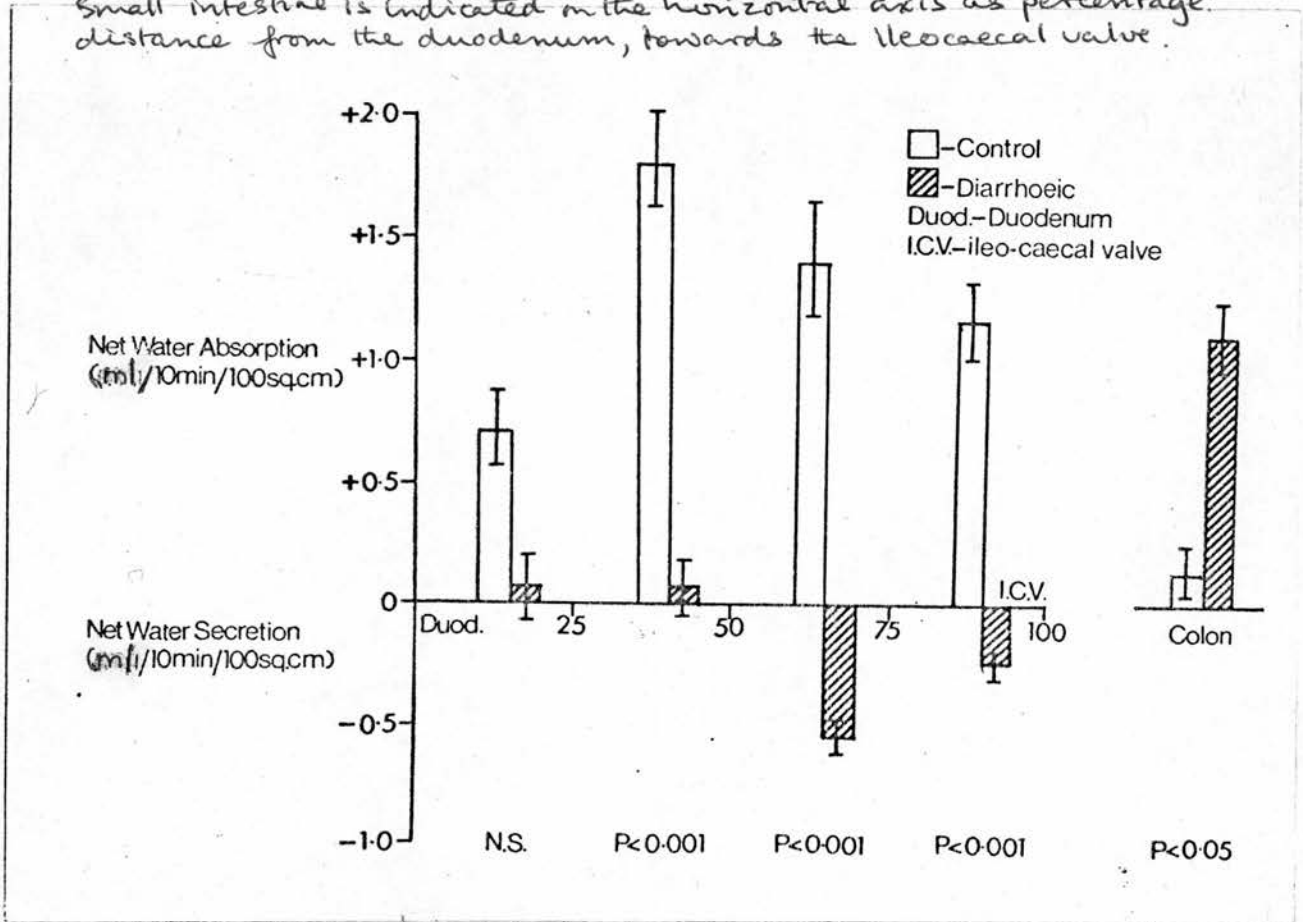


Figure 28. The Effect of *E. coli* Toxin on Net Sodium Absorption.

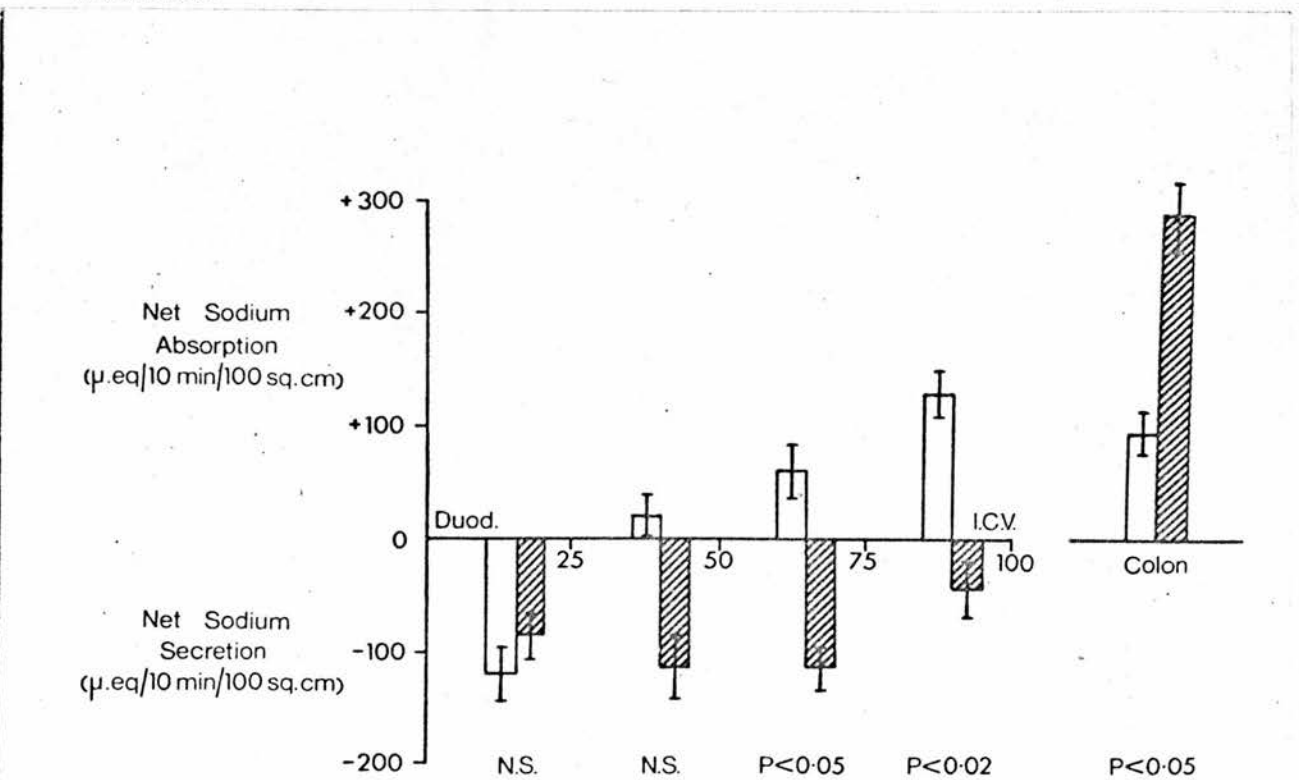


Figure 29. The Effect of E. Coli Toxin on Net Potassium Secretion.

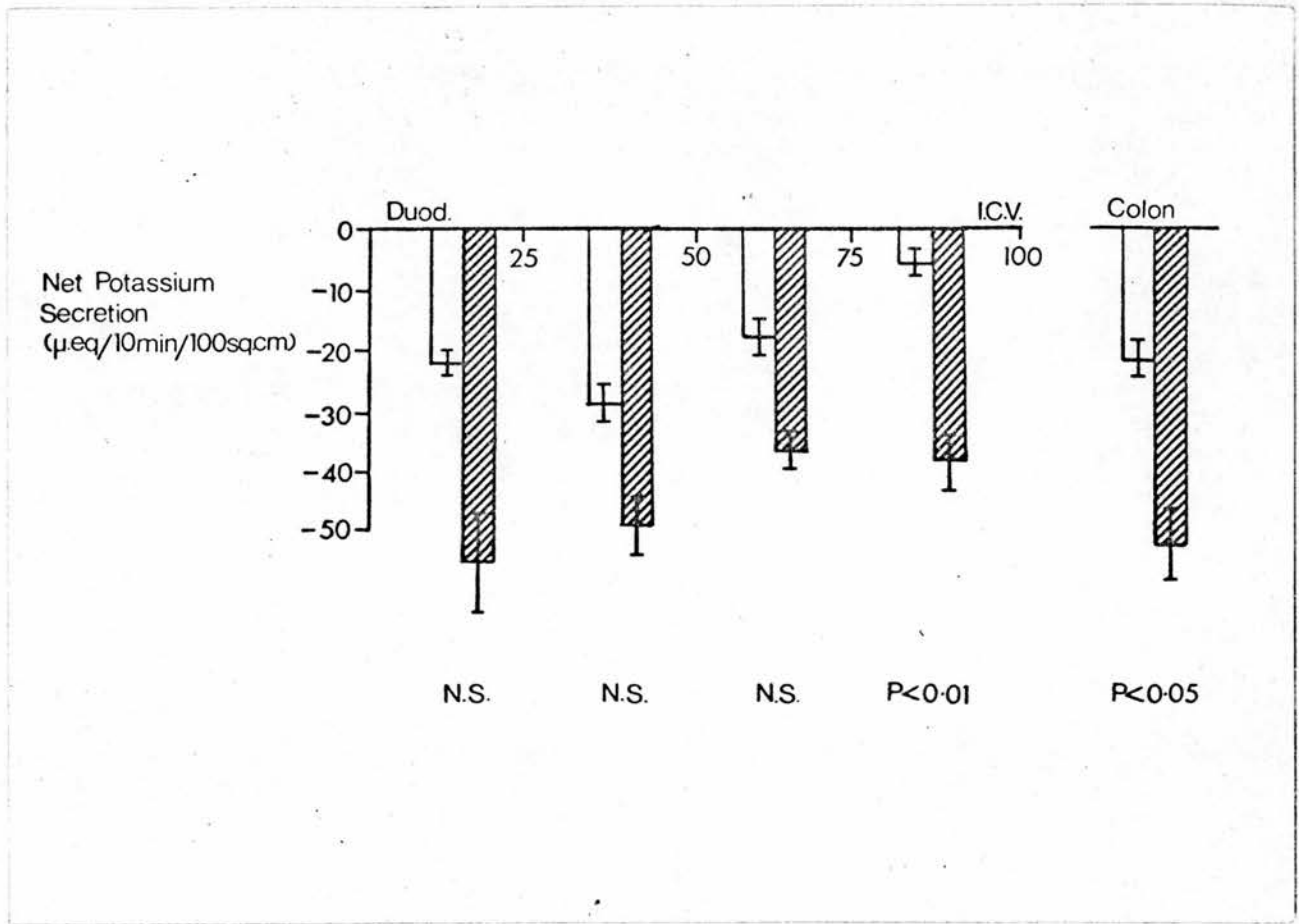


Figure 30. The Effect of E. Coli Toxin on Net Chloride Absorption.

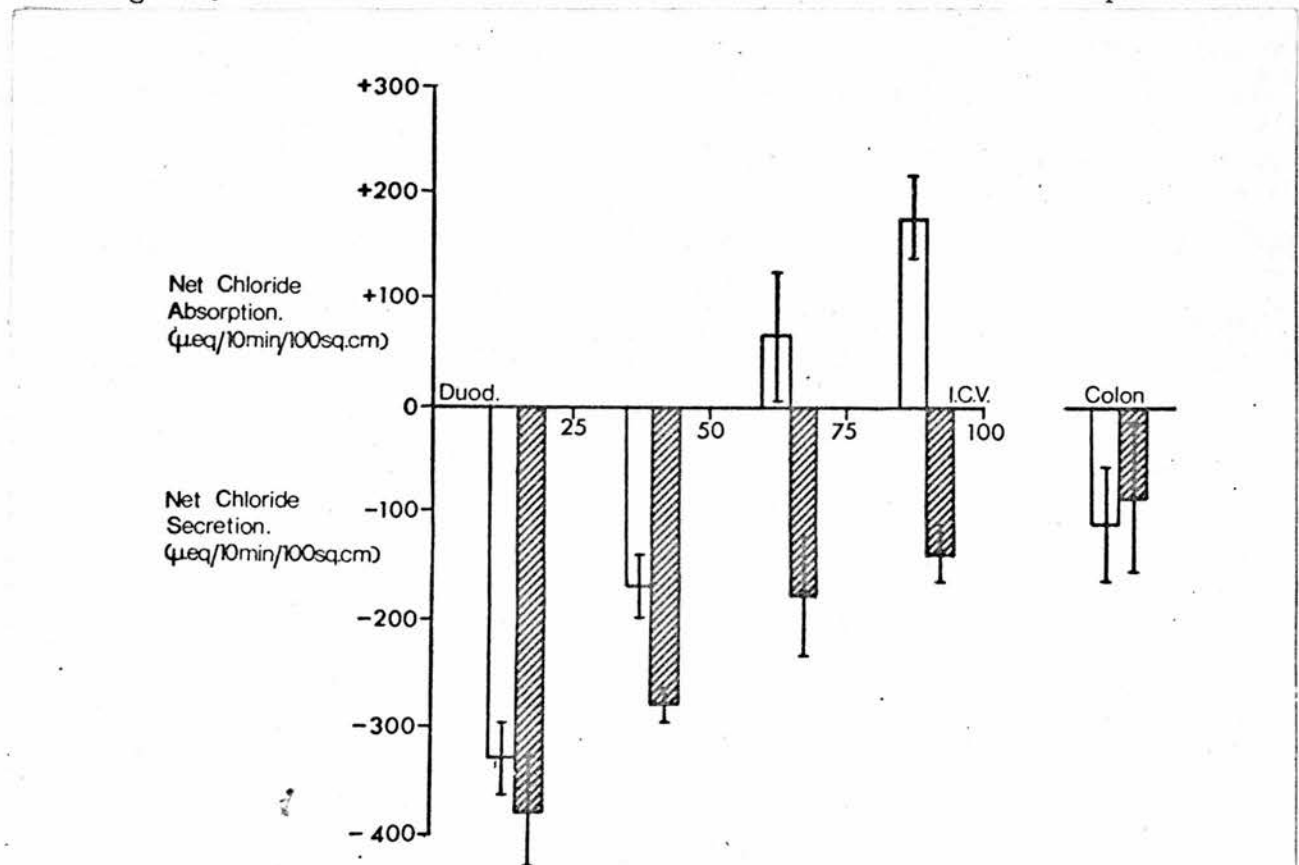
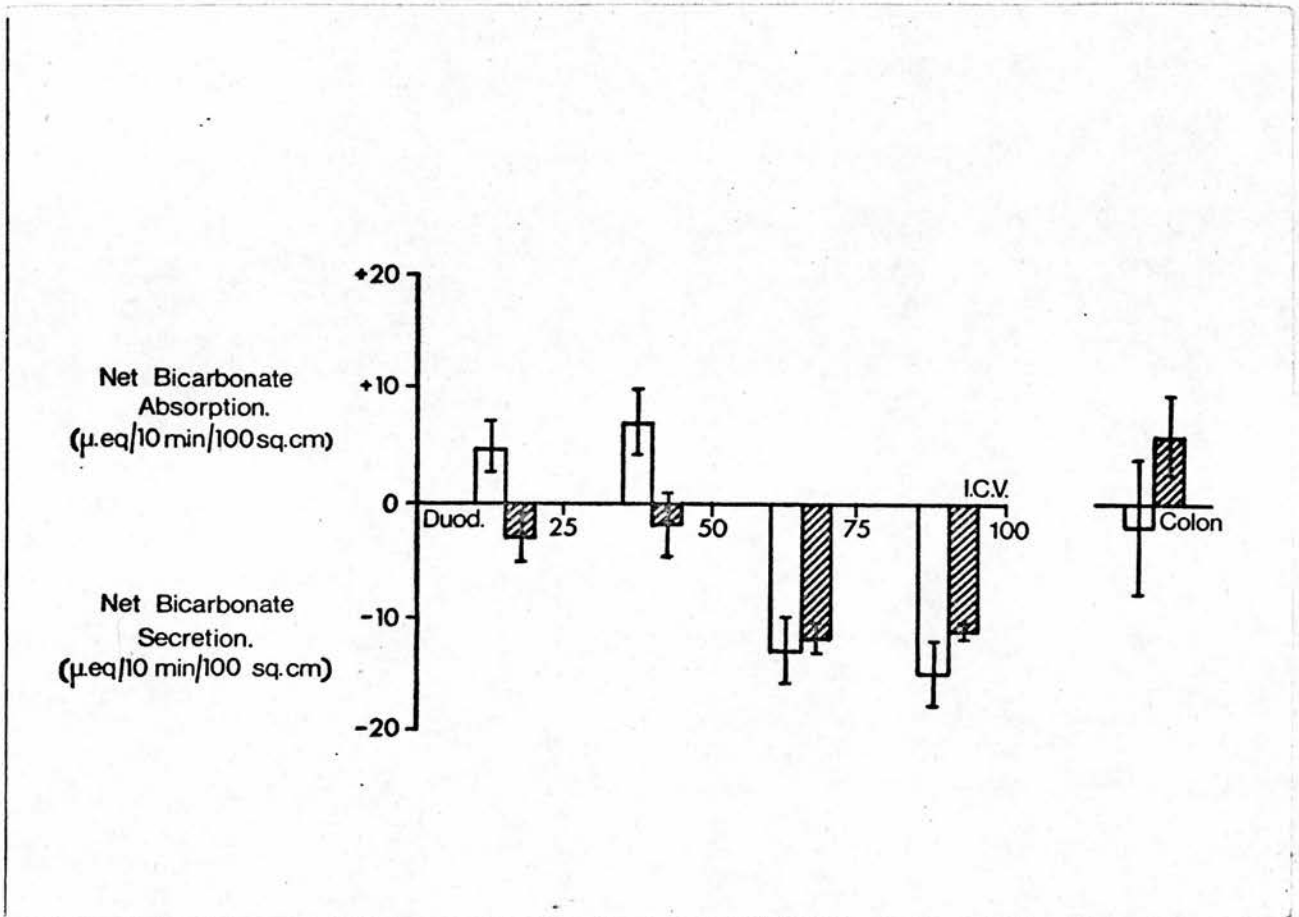


Figure 31. The Effect of *E. coli* Toxin on Net Bicarbonate Absorption.



General Conclusion (Section 2)

The evidence presented in this section of the thesis suggests that like cholera toxin, *Escherichia coli* heat stable toxin initiates fluid secretion and electrolyte loss in the small intestine by a mechanism independent of prostaglandin production and mediation.

Intestinal washings from infected and healthy piglets did not show any marked difference in prostaglandin content. Thus no overflow mechanism involving prostaglandin biosynthesis and release appeared to be operational (Experiment 2). *Escherichia coli* toxin itself was devoid of prostaglandin-like activity (Experiment 1) thus no direct prostaglandin step was involved in the initiation of secretory responses. The synthesis of PGE_2 and $\text{PGF}_{2\alpha}$ was no greater in intestinal tissue taken from *E. coli* toxin infected animals than in samples of calves (Experiment 3). This suggested that prostaglandin biosynthesis in the intestine did not play an integral part in the intestinal reaction to toxin administration. These results were further supported by experiments in which prostaglandins (PGE_1 and PGA_1) were administered intraluminally to piglets. In these experiments no net secretion of fluid was observed. Bicarbonate ion secretion was enhanced. These effects did not resemble those seen following toxin administration either in vivo or in vitro to piglet intestine (Experiment 5).

Prostaglandins are not likely to be mediators of the effects of *E. coli* heat-stable toxin in neonatal calves. Intra-arterial infusion of PGE_2 , PGA_1 and $\text{PGF}_{2\alpha}$ did not mimic the effects of toxin administration on fluid and electrolyte loss in the small bowel (Experiment 6).

It is therefore concluded that *Escherichia coli* heat-stable toxin does not initiate fluid secretion in the gastrointestinal tract by stimulating the biosynthesis and release of prostaglandin compounds.

General Discussion

In the introduction it was suggested that there were two possible mechanisms whereby cholera toxin and other bacterial toxins might initiate fluid secretion in the small intestine via a prostaglandin or prostaglandin-like mediator (see Introduction, Figure 4). The first pathway implicated a prostaglandin of bacterial origin. The second was dependent on host biosynthesis in response to the toxin stimulus. It has been possible to demonstrate that neither of these hypotheses is valid and that prostaglandins or prostaglandin-like compounds are not implicated in either experimentally-induced cholera or *E. coli* diarrhoea.

Toxin and Prostaglandin

The prostaglandin content of cholera enterotoxin and heat-stable *Escherichia coli* enterotoxin was determined. No prostaglandins were detected in cholera toxin prepared from *Vibrio cholerae* strain 569B (Wyeth lot 002 toxin). The lower limits of the assays were 12.5 ng, 25 ng and 90 ng per gram solid weight of toxin for PGE_2 , $\text{PGF}_{2\alpha}$ and PGA_1 equivalents respectively. The dose of toxin used to initiate choleraic fluid loss in cats was 1 g while the amount used in the rabbit perfused ileal loop model was 100 mg solid weight toxin. Thus the total prostaglandin administered to rabbit intestine was no more than 1.25 ng PGE_2^- , 2.5 ng $\text{PGF}_{2\alpha}^-$, and 9.0 ng PGA_1 -like material. No prostaglandin-like activity was found in two of the three batches of *E. coli* toxin prepared from *E. coli* strain B44 and the respective medium controls. The limits of detection were 10 ng PGE_2 equivalents, 25 ng $\text{PGF}_{2\alpha}$ equivalents and 60 ng PGA_1 equivalents per 60 ml toxin solution and sterile broth. In The third sample of toxin solution contained

10 ng PGE_2 -like and 100 ng $\text{PGF}_{2\alpha}$ -like material but no PGA_1 -like activity was detected. The corresponding control medium was also observed to contain PGE_2 - and $\text{PGF}_{2\alpha}$ -like activity amounting to 12 ng and 100 ng per 60 ml fluid respectively. No PGA_1 -like substances were found. The limit of assay for PGA_1 -like material in both the sterile broth and active toxin solution was 60 ng per 60 ml fluid. The volume of toxin used to challenge intestinal loops was 1.5 ml in piglet experiments. Thus the maximum total dose of prostaglandin administered was 0.3 ng PGE_2 equivalents, 2.6 ng $\text{PGF}_{2\alpha}$ equivalents and 1.5 ng PGA_1 - equivalents. Sterile broth does not induce fluid secretion in the small intestine and thus it could be concluded that the amounts of prostaglandin administered in the active toxin solution were insufficient to account for the fluid and electrolyte loss caused by the toxin.

It was considered unlikely that $\text{PGF}_{2\alpha}$ or a PGF -like compound might have been responsible for fluid production due to bacterial toxin infections as $\text{PGF}_{2\alpha}$ had been observed to enhance plasma protein loss from canine jejunum in association with the extrusion of electrolytes and water (Pierce et al, 1971). Choleraic fluid and *E. coli* diarrhoeic fluid are not rich in protein matter (Weaver et al, 1948; Pierce et al, 1971; Pierce & Wallace, 1972). The situation encountered in the intestine following local bacterial enterotoxin administration resembles the excretory activity of the kidney in that only salt and water is lost from the organ (see e.g. Davson & Eggleton, 1968). PGE and PGA compounds stimulate fluid and electrolyte extrusion by the kidney (Herzog, Johnston & Lauler, 1967; Davis, 1971; Fulgraff, Brandenbusch & Meiforth, 1972, 1973). Thus it seemed that the most likely candidates as mediators of the effects of the

enterotoxins were prostaglandins of the E and A series.

The absence of such compounds in cholera toxin preparations confirmed the work of Horton & Collee (unpublished observations). Similar findings have since been reported by Bedwani (personal communication) who was unable to detect any PGE_2 -like activity in extracts of crude Wyeth lot 001 cholera toxin and in highly purified toxin. In all of these investigations biological assay was used to estimate prostaglandin-like activity.

Pierce and his associates (Pierce et al, 1971) demonstrated that microgram quantities of prostaglandins of the E, F and A series were required to elicit a secretory response from the intestinal mucosa when given by either intra-arterial or local intraluminal infusion to dogs. Thus it is highly unlikely that the prostaglandin-like activity detected in any of the toxin preparations used to stimulate fluid production in experimental animals could have been responsible for the secretory stimulant properties of the toxin solutions.

It was also difficult to reconcile the long duration of the effects of cholera toxin with the brevity of those of prostaglandins. In the intestine it has been observed in vivo and in vitro that the stimulation of fluid secretion and adenylyl cyclase activity by PGE_1 is rapidly terminated following the removal of this compound. Removal of cholera toxin does not result in a rapid cessation of enhanced activity (Pierce et al, 1971; Kimberg et al, 1971; Al Awqati & Greenough, 1972). The evanescent nature of the intestinal secretory stimulant activity of *Escherichia coli* toxin was compatible with this hypothesis however (Guerrant, Ganguli, Casper, Moore & Pierce, 1973).

The absence of appropriate polyunsaturated fatty acids in

cholera toxin filtrate (Kaur et al, 1970) and the fact that purified cholera toxin is a high molecular weight protein (Finkelstein, 1972) also argued against a direct prostaglandin link. Furthermore it was impossible to give a logical and valid explanation of results like those of Kimberg and his associates who demonstrated that PGE_1 and cholera toxin were both able to elicit maximal stimulation of adenylyl cyclase activity equivalent to that observed in the presence of PGE_1 and toxin alone respectively, when the tissue had been previously exposed to the other agent (Kimberg et al, 1971). Sharp and Hynie have claimed to have enhanced the response of adenylyl cyclase activity to PGE_1 by cholera toxin pretreatment (Sharp and Hynie, 1971) but the actual prostaglandin-elicited part of the response was not significantly greater than that observed in the absence of the active toxin. Had toxin activity been directly associated with prostaglandin compounds, then an inhibitory effect of each of these substances on the other would have been expected.

Furthermore, it is possible to dissociate the stimulant properties of cholera toxin and *Escherichia coli* toxin from those of PGE compounds by investigating adenylyl cyclase systems other than those found in the gastro-intestinal tract. Both cholera toxin and *Escherichia coli* toxin preparations stimulate fat cell adenylyl cyclase activity (Curlin & Chen, 1971; Hewlett & Greenough, 1972; Greenough, Pierce & Vaughan, 1970; Hewlett, Guerrant, Evans & Greenough, 1974) but do not inhibit the lipolytic actions and increase in adenylyl cyclase activity induced by other hormones, e.g. adrenaline (Katz & Greenough, 1972; Hewlett et al, 1974). In contrast prostaglandins inhibit adenylyl cyclase activity which is hormonally induced (Steinberg, 1967; see Reviews of Horton, 1972

and Karim, 1972) but do not stimulate lipolysis directly in isolated fat cells. Furthermore the time course of adenylyl cyclase activation by bacterial toxins is different to that of prostaglandin-induced activity, there being marked discrepancies in the latency of onset, duration and rapidity of termination of effects (see Sharp, 1973).

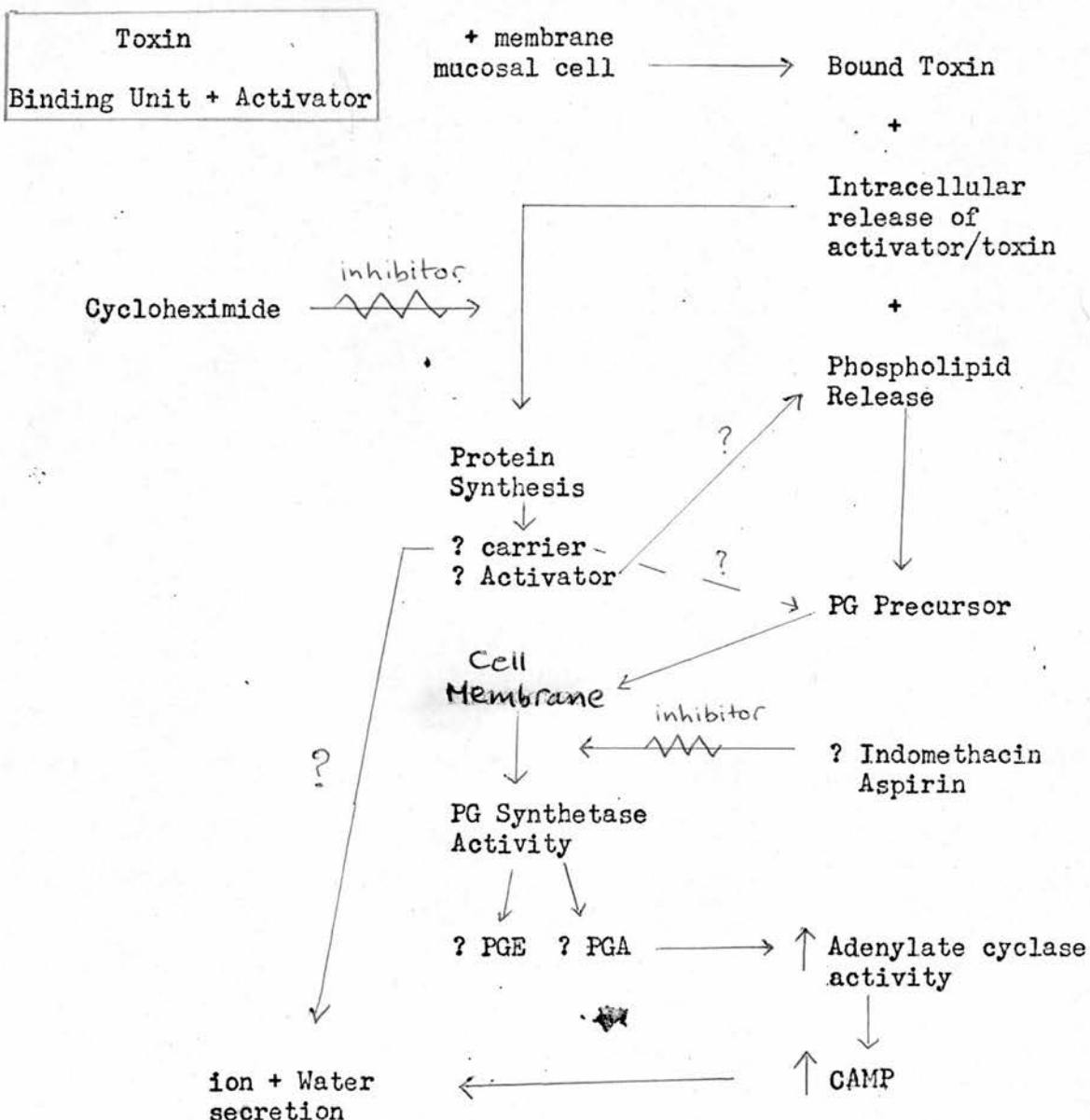
Finally it has been demonstrated by Pierce (1973) that there are separate intestinal mucosal cell receptors for cholera enterotoxin and *E. coli* heat-stable enterotoxin. Gangliosides, and natural cholera toxoid inhibit the binding of active cholera toxin but not that of *E. coli* toxin. The possession of discrete receptor sites by the mucosal cell for each of these toxins implies that a common direct pathway reliant on prostaglandin-like moieties in the toxins is unlikely to be responsible for the fluid loss and that there are probably two activating mechanisms in the cell wall, one for each toxin.

Thus there is little or no evidence to support the statement that toxin release of prostaglandin-like substances is responsible for the secretory stimulant properties of either *Vibrio cholerae* or *Escherichia coli* enterotoxins.

Escherichia coli toxin, Cholera toxin and Intestinal prostaglandins

Much of the data in the literature could however be reconciled with the second postulated mechanism of prostaglandin-dependent toxin-induced fluid production. The relevant part of Figure 4 is reproduced in Figure 32 for clarity. The inability of PGE_1 -induced maximal adenylyl cyclase activity to approach the levels of enhanced activity achieved with cholera toxin and *E. coli* toxin (see Kimberg et al, 1971; Kimberg, Field, Gershorn and Henderson, 1974; Evans et al, 1972; Guerrant et al, 1973) could be accounted for by the

Figure 24



inability of sufficient quantities of prostaglandin to escape metabolism and reach the adenylyl cyclase regulator sites. Penetration would not be a problem for intracellularly synthesized prostaglandin. The difference in time course of adenylyl cyclase activation and duration of effects between cholera toxin and *E. coli* toxin were also explicable. Cholera toxin consists of a binding and an activator subunit. *E. coli* heat-stable toxin on the other hand is a relatively low molecular weight compound (see Introduction). It is

possible that the prolonged latency observed with cholera toxin is attributable to the delay in the release of the activator fraction and initiation of prostaglandin biosynthesis. This latter process may well be an indirect protein-dependent step, the protein itself requiring to be synthesized de novo as cycloheximide interrupts the toxin-initiated processes which culminate in fluid secretion (Serebro et al, 1969; Grayer et al, 1970; Moritz et al, 1972). Tenuazotic acid, also a protein synthesis inhibitor (Moritz & Womelsdorf, 1973) has similar effects. ⁽¹⁹⁷⁴⁾ Cuatrecasas has shown that the binding of cholera toxin to cell membranes is a two stage reaction: there is an initial rapid attachment of the toxin to the membrane surface; this is followed by a slower reaction in which some change in the toxin configuration relative to the membrane receptor occurs which is irreversible, stabilizes the toxin-receptor binding and results in the intracellular extrusion of the activator subunit (Cuatrecasas, 1973a). Similar studies have not been performed using *E. coli* toxin. It has however been shown by Hewlett, Guerrant, Evans and Greenough (1974) that washing of isolated fat cells following exposure to *E. coli* toxin limits the toxin-induced stimulation of adenylyl cyclase activity and glycerol release. This together with the work of Pierce suggests that *E. coli* toxin may only be weakly bound to the mucosal cell membrane and initiate secretory activity from that membrane site rather than by a more direct intracellular action similar to cholera toxin. Leitch and Glinkson observed changes in the lipid content of mucosal cell membranes (see Introduction). Phospholipids are storage sites for prostaglandin precursors. The fate of the phospholipid released from the membrane has yet to be established. The loss of these compounds during exposure of the intestinal mucosal cell to cholera toxin might

indicate that prostaglandin biosynthesis was indeed taking place within the mucosal cell.

The discrepancy between the doses of intra-arterially infused prostaglandins and of intraluminally administered compounds required to induce fluid secretion in canine small bowel could in part be accounted for in terms of the above hypothesis. Firstly the adenylyl cyclase responsible for ion and water extrusion is not located in the brush border membrane but in the lateral and basal membranes (see Sharp, 1973). Thus there is a much greater ease of access to these sites for intra-arterial prostaglandins than for intraluminally applied compounds, which have to overcome uptake and metabolism processes in sufficient quantities in addition to actually reaching the target adenylate kinase.

No explanation could however be given for the results obtained on maximal stimulation of adenylyl cyclase activity by PGE_1 or cholera toxin (Kimberg et al, 1971) nor for Sharp and Hynie's results (Sharp & Hynie, 1971). That these two reports did not concur with the remainder of the evidence in support of this hypothesis was insufficient grounds for rejecting the hypothesis outright. However direct evidence of prostaglandin participation was required in order to prove it.

The results obtained in this investigation from direct observations on the release, and biosynthesis of prostaglandin-like compounds did not support the theory of prostaglandin-mediated, toxin-induced fluid loss in the small bowel. Prostaglandin-like material was present in fluid secreted into the lumen of ligated ileal loops in rabbits and was characterized by solvent extraction, purification and silicic acid column or silica gel thin layer chromatography followed by biological assay. Intestinal washings

from *E. coli* toxin-infected piglets were also found to contain prostaglandin-like activity. In the rabbit PGE-like and PGA-like material was detected both in samples collected 8 to 12 hours after toxin administration and in those obtained at 22 to 24 hours after challenge. The amounts present in the 22 to 24 hour samples were greatly in excess of those in samples from the experiments of shorter duration. This markedly enhanced release corresponded in time with the development of extensive haemorrhagic necrosis of the ligated, toxin-treated loop wall and with considerable distension of the loop itself.

When a perfused ileal loop model was used instead of this ligated loop model it was apparent that the prostaglandin release induced in the ligated loop was mainly attributable to the trauma experienced by the intestinal tissue. Prostaglandin E and F-like compounds were present in low concentrations both in washings from *E. coli* toxin-infected animals and in control, healthy uninfected piglets. This suggested that the prostaglandin release was largely due to the washing procedure itself. However, in these experiments in which toxin-challenged and control tissues produced prostaglandins it is impossible to differentiate the prostaglandin which is synthesized as a result of tissue injury and that which represents an overflow from the intracellular secretory processes. Such an overflow mechanism would be expected to contribute picogram amounts of prostaglandin to the total output. Thus in the absence of a detecting system which permits differentiation of prostaglandin output from the various sources, it is unlikely that in vivo direct measurement of release can afford much conclusive evidence either in favour or against the hypothesis.

This problem can be overcome to a certain extent by investigating the synthesizing capacity of toxin-treated tissue. If, as it was postulated, the toxins are responsible for augmenting prostaglandin biosynthesis intracellularly it should be possible to measure the difference between control and infected tissue output in in vitro situations where the trauma is more easily controlled and can be adjusted so that toxin-treated and control tissues are studied in identical conditions. The difference in output in this situation then represents the effect of the toxin per se. This approach was used with both cholera toxin and E. coli-treated intestinal tissues, appropriate controls also being carried out. It was observed that highly purified cholera toxin had no effect on prostaglandin output by intestinal tissue. Thus it would seem that little change in the ability of the gut to synthesize prostaglandins is wrought by the pure form of toxin. Bedwani has since confirmed this observation (Bedwani, 1974). Crude cholera toxin was, in his hands, found to stimulate prostaglandin output slightly but the effect was also observed with crude boiled, inactive cholera toxin. Thus it is apparent that impurity of the toxin itself can sometimes lead to enhanced prostaglandin biosynthesis. Kimberg, Field, Gershorn & Greenough (1974) have confirmed that prostaglandin output is not augmented by purified cholera toxin. There was no significant difference in the levels of PGE (converted to PGB) and $\text{PGF}_{2\alpha}$ as determined by radio-immunoassay.

The incubation period used for these investigations was 3 hours. The increase in adenylyl cyclase following cholera toxin administration is not immediate, the latent period being approximately 1 hour (Field et al, 1969; Chen, Rohde & Sharp, 1972) but the effects are long lasting. Thus by using this 3 hour period it

should have been possible to detect some change in the amount of prostaglandin being produced if these substances were responsible for the rise in adenylyl cyclase activity. Because control samples were also studied the effect of metabolism of prostaglandins in the cell was not investigated, ~~the~~ argument being that metabolism in the cells must have been proceeding at similar rates in toxin-treated and control tissues if a net effect on adenylyl cyclase activity and in vivo fluid secretion was observed. *Escherichia coli* toxin on the other hand does not have a long action on either adenylyl cyclase activity or fluid output (Guerrant et al, 1973). Activity falls back to control levels very rapidly after toxin is removed from contact with the intestine and even in the continued presence of toxin, the levels of adenylyl cyclase activity fall to control levels after approximately 6 hours. In the in vitro investigations with this toxin a one hour period was used, thereby ensuring that a period in which enhanced adenylyl cyclase activity and fluid secretion would be expected to occur, was being investigated. The lack of effect of the *E. coli* heat-stable toxin therefore indicated that a prostaglandin-dependent mechanism was not likely to be responsible for the pathological effects of the toxin.

Thus the evidence from in vitro investigations confirmed the results of in vivo experiments and gave additional weight to the conclusions drawn from these experiments. It now seems very unlikely that prostaglandins were of prime importance in toxin-induced fluid secretion.

An alternative approach to the problem was to study the effects of prostaglandin synthesis inhibitors on the secretion of fluid in the small bowel in response to toxin administration.

Two such compounds were selected namely, indomethacin and aspirin. It was observed that systemic administration of indomethacin in doses of 10 to 100 mg per kg body weight and local application of a 600 μ g dose to the small intestine in cats failed to depress the secretory response of the intestine to subsequent challenge with crude cholera toxin. Indomethacin given chronically over a 3 day period, or as acute doses also failed to prevent fluid production in rabbit models. The doses used were 10 to 20 mg per kg in acute experiments while in chronic treatment schedule doses of 40 mg per day were employed. Aspirin in doses of 25 mg per kg did not modify fluid secretion in the terminal ileum of cats, when administered intravenously before toxin challenge. Systemic administration of indomethacin in doses of 1 to 5 mg per kg body weight in dogs has been observed to abolish renal prostaglandin output (Herbaczynska-Cedro and Vane, 1972). Davis (Davis, 1972) made similar observations in rabbits following intravenous injections of 10 mg/kg body weight. In guinea-pigs, a species which has a plasma half life for indomethacin of twenty minutes (Hucker et al, 1966), a dose of 20 mg per day of indomethacin can prolong the oestrous cycle in guinea-pigs (Poyser, 1972) which also suggested that prostaglandin biosynthesis was being inhibited.* Oral administration of indomethacin (1mg) and aspirin (10 mg/kg) has been claimed by Somova (1972) to reduce prostaglandin output in hypertensive cat and rat kidneys to the levels observed in normotensives. Ferreira, Herman & Vane (1972) demonstrated that indomethacin in doses of 1-10 μ g/ml inhibited prostaglandin release from isolated strips of rabbit jejunum. Thus it was highly unlikely that the doses used to treat cats and rabbits were having no effect on prostaglandin synthetase activity.

viz. Indomethacin prolongs oestrus; endogenous prostaglandin F_{2a} is known to terminate oestrus.

Bedwani has confirmed that indomethacin does not abolish the secretory stimulant properties of cholera toxin in rabbits in vivo (Bedwani, personal communication). It was reported by Fink and Katz (1972) that aspirin given as a single intravenous injection in a dose of 25 mg per kg body weight in cats is capable of inhibiting the secretion of fluid by the jejunum following exposure to cholera toxin. The toxin used in these studies (Fink and Katz's work) was Wyeth lot 001 toxin. As demonstrated by Bedwani, it was highly unlikely that the prostaglandin content or release of prostaglandin caused by the toxin could have been responsible for the fluid losses encountered (Bedwani, personal communication). As previously stated, Pierce and his associates demonstrated that the concentration of prostaglandins, whether PGE_1 or PGA_1 , required to induce fluid production in the jejunum in dogs was of the order of micrograms per minute (Pierce et al, 1971). The only factors which might have influenced the results and hence the discrepancy between Fink and Katz's work and the observations reported in this thesis was the strain of cat and perhaps the insensitivity of the jejunum to indomethacin versus its sensitivity to aspirin.

That the jejunum should be less sensitive to the effects of indomethacin than to those of aspirin seems rather unlikely. In the wide range of synthetase inhibitors investigated to date, there is not one in which aspirin appears to be a more effective prostaglandin synthetase inhibitor than indomethacin. Even with brain synthetase which is remarkably sensitive to paracetamol, the same order of potency is adhered to for indomethacin and aspirin (see Flower, 1974). Thus it appears that species differences are most likely to be responsible for the observed effects of indomethacin in the cat.

A further report of inhibition of choleraic fluid production by anti-inflammatory agents was made by Jacoby and Marshall (1971). The species investigated was the rat. Increase in stomach and duodenal weight were used as the index of fluid secretion. The results of these experiments were not very convincing however.

There have been at least two reports of the failure of indomethacin to inhibit the effects of cholera toxin in other experimental situations. In fat cells, Illiano and Cuatrecasas were unable to prevent cholera toxin-induced lipolysis by incubating isolated fat cells with indomethacin in concentrations up to 100 μg per ml. Similar inability to modify the lipolytic response was seen with sodium salicylate in concentrations as great as 200 $\mu\text{g}/\text{ml}$ (Illiano & Cuatrecasas, 1971; Cuatrecasas, 1973c). In this model it should be remembered that prostaglandins do not stimulate lipolytic activity but antagonise the effects of other hormones (see earlier part of discussion). The work of Bourne (1971) also supports the conclusions drawn from the results presented in this thesis. Bourne showed that indomethacin did not inhibit cholera toxin-induced stimulation of adenylyl cyclase activity in neutrophil cells. These results are comparable to the recent observations made by Kimberg and his associates on rabbit mucosal adenylyl cyclase activity (Kimberg et al, 1974).

The negative findings reported in this thesis were supported by the demonstration of the ability of levels of indomethacin circulating in cat plasma to inhibit in vitro prostaglandin biosynthesis by 80% or more, the dose of indomethacin injected being either 10 mg/kg or 50 mg/kg. Thus the case against prostaglandin mediation of the secretory effects of cholera toxin was

strong. Similar studies have not been undertaken in either the calf or pig. It has, however, been observed that indomethacin, when applied locally to piglet intestine, does not inhibit fluid secretion elicited by *E. coli* toxin (McLaughlin, personal communication) and thus it does seem likely that prostaglandins are not primary instigators of fluid production in *E. coli* diarrhoea either.

It was apparent from the results of the in vivo prostaglandin release experiments, the in vitro prostaglandin biosynthesis studies and the inability of indomethacin to inhibit fluid secretion in the in vivo rabbit and cat experimental models of cholera, that cholera toxin did not depend on a prostaglandin-mediated step to initiate fluid and electrolyte secretion into the intestinal lumen. The evidence against *Escherichia coli* enterotoxin fluid loss being attributable to prostaglandin release was also strong, and was further supported by additional studies on ion and water fluxes in the small intestine following prostaglandin administration. It was established that *E. coli* toxin did not produce the same qualitative effects on ion and water movement as prostaglandins in the intestine of piglets and calves. This therefore eliminated the possibility that *E. coli* toxin did operate through a prostaglandin (or prostaglandin-like) moiety.

In piglet small intestine *E. coli* heat-stable toxin depresses glucose uptake, and effects on water and sodium fluxes are thought to be secondary to the depression of glucose uptake (Bywater & Jefferies, unpublished observations). PGA_1 and PGE_1 on the other hand had pronounced effects on bicarbonate ion movement. The effect of PGE_1 was much more marked than that observed with PGA_1 . At these dose levels glucose absorption was slightly but not significantly depressed. Prostaglandins have been observed to

inhibit glucose uptake in the small intestine of man (Matuchansky & Bernier, 1973) but the dose of PGE_1 required is substantial - being $0.9 \mu\text{g/kg/minute}$ infused into the jejunum. Coupar and McColl (1972) observed a similar effect with PGE_1 , E_2 and $\text{F}_{2\alpha}$ in rat small intestine in vivo. In these experiments the dose required was $1.4 \times 10^{-4}\text{M}$ this is equivalent to approximately $45 \mu\text{g/ml}$. Cholera toxin does not influence glucose absorption either in human intestine in vivo (Pierce et al, 1968) or in vitro (Iber et al, 1969). Indeed glucose solutions antagonize the effects of cholera diarrhoea in the small intestine. In rabbit intestine glucose absorption is not impaired by cholera toxin (Love, 1969) whereas *E. coli* toxin has been observed to depress glucose uptake (Whipp & Moon, 1972). Thus there would appear to be a fundamental difference between the effects of heat-stable *E. coli* toxin and cholera toxin. This is, however, likely to be a species related phenomenon as calf intestinal uptake of glucose is not sensitive to heat-stable *E. coli* toxin (Bywater, 1970).

In the calf experiments prostaglandins were administered via the superior mesenteric artery. Neither PGE_2 nor $\text{PGF}_{2\alpha}$ produced any marked effect on electrolyte and water transport. PGA_1 however was found to enhance bicarbonate ion secretion. This prostaglandin did not cause any significant change in any other parameter measured in these experiments.

It was notable that prostaglandins of the E series were more active than those of the A series in stimulating bicarbonate ion secretion in the small intestine following intraluminal administration whereas PGA_1 was active in intra-arterial infusion in contrast to PGE_1 . Although species differences may account for these findings it would be interesting to determine whether the

intraluminal-intra-arterial difference might not reflect alteration of PGE compounds during uptake by the intestinal mucosa. Furthermore in vitro cholera toxin has been shown to stimulate chloride ion secretion and reduce sodium absorption, an effect mimicked by prostaglandins (Al Awqati & Greenough, 1973; Field, Fromm, Al Awqati & Greenough, 1972). In vivo it is the bicarbonate ion which is the major component of anion secretion (Love, 1966). Recent work of Powell, Binder and Curran (1973) does suggest that chloride ion secretion in vitro may be dependent on the addition of toxin in vivo as in these in vitro experiments where tissues were not exposed to cholera toxin in vivo, bicarbonate ion secretion was observed. In human intestine bicarbonate ion, the residual ion flux, is also enhanced (Rohde & Anderson, 1973). It therefore seems feasible that, since prostaglandins can enhance bicarbonate ion secretion in vivo, the control of bicarbonate ion secretion may be due to the balance of prostaglandin activity in the intestinal mucosa. Prostaglandins have been shown to be present in the mucosal layer of the intestine and as yet no function has been attributed to them in this situation.

Having thus excluded prostaglandins as mediators of cholera toxin and E. coli heat-stable toxin-induced fluid secretion, the problem still remains as to what does act as a mediator within the mucosal cell. Polypeptides are found in the mucosa of the small intestine. Barbezat and Grossman (1971) have shown that vasoactive intestinal peptide (VIP), gastric inhibitory peptide (GIP) and glucagon all stimulate fluid secretion in canine jejunum. Glucagon stimulates adenyl cyclase activity in rat fat cells (Butcher & Baird, 1970). This ability is shared by both V. cholerae and E. coli toxins (Hewlett, Guerrant, Evans & Greenough, 1974) but

neither of these toxins abolishes the sensitivity of fat cell adenylyl cyclase to hormones (Vaughan, Pierce & Greenough, 1970; Katz & Greenough, 1972). In fact it was demonstrated by Hewlett and his associates that adrenaline sensitivity was actually increased (Hewlett et al, 1974). Adrenaline and glucagon in fat cells activate the same receptor (Birnbaumer & Rodbell, 1969). Thus it seems doubtful whether endogenous hormone production could be responsible for the effects of either enterotoxin.

It may be that fragments of the toxin themselves are the mediators. Raskova (1974) has suggested that cholera toxin modifies the regulator unit which is responsible for basal activity and not the hormone sensitive unit. If this hypothesis is valid then not only would it account for the additive effects seen with cholera toxin and prostaglandins (Kimberg et al, 1971) but it would also explain the apparently anomalous findings of Sharp and Hynie (1971). Recent work by Kantor, Tao and Kiefer (cited in Kantor, Tao & Gorbach, 1974) suggests that a similar situation may exist for *Escherichia coli* toxin and PGE_1 , as kinetic evidence has been obtained which indicates that there are separate receptor sites on intestinal adenylyl cyclase for each of these agents.

It is also clear from the results presented in this thesis that the wide scale use of aspirin and other prostaglandin synthesis inhibitors as a means of treating cholera (Bennett, 1971) and other similar bacterial diarrhoea is unlikely to be successful. Recently the alkaloid of *Berberis Anistata* has been found to reduce the diarrhoeic effects of *Vibrio cholerae* and non-vibrial organisms (Dutta & Panse, 1962; Purekit & Sabhadra Rao, 1969;

Lahiri & Datta, 1967). It is thought to produce its effects by altering the fatty acid composition of the infective organism and by stimulating protein and lipid synthesis (Modak, Modak & Venkataraman, 1970). It could well be that the development of synthetic compounds based on this alkaloid could provide a potent, rapidly effective drug against vibrio cholerae and other (similar) organisms like *Escherichia coli*.

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THE FAILURE OF INDOMETHACIN TO MODIFY THE RESPONSE OF CAT SMALL INTESTINE TO CHOLERA ENTEROTOXIN

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M. J. DLUGOLECKA: *The failure of indomethacin to modify the response of cat small intestine to cholera enterotoxin.* Pol. J. Pharmacol. Pharm. 1974, 26, 93—100.

In a series of 21 cats in which the terminal small intestine was challenged with cholera toxin, the effect of two prostaglandin synthesis inhibitors and anti-inflammatory drugs were studied. Neither indomethacin in doses of 10, 50 and 100 mg/kg by intravenous injection nor in a dose of 600 µg/loop when administered locally, nor aspirin intravenously injected in a dose of 25 mg/kg were able to abolish the response of the intestine to the toxin. It is concluded that prostaglandins are not likely to be mediators of the fluid loss observed in the terminal small intestine.

Preliminary experiments in rabbits have indicated that indomethacin chronically administered prior to cholera toxin challenge does not inhibit fluid accumulation in ligated intestinal loops but is capable of preventing haemorrhagic necrosis. This report concerns experiments in the cat using perfused intestinal loops similar to those of Fink and Katz [5] in cats and of Pierce et al [9] in dogs. In the present study animals were pretreated with either indomethacin or aspirin. The effect of these drugs on the response to cholera enterotoxin in the small intestine is reported.

METHODS

Twenty-one young cats and kittens weighing 1.3—3.75 kg were used in these experiments. Anaesthesia was induced with pentobarbitone sodium, 40 mg/kg and maintained by continuous intravenous infusion of pentobarbitone sodium in saline (6 mg/ml) at rates varying between 0.075—6.6 ml/hr. The anaesthetic solution contained heparin 100 iu/ml. Tracheostomy was performed and animals artificially ventilated if respiration became unsatisfactory. The right common carotid artery was cannulated for blood pressure recording on a Grass polygraph. The right femoral vein or external jugular vein was cannulated to administer the anti-inflammatory drugs. All vascular cannulae were primed with heparin 100 iu/ml before use.

A mid-line abdominal incision was made and the terminal small intestine exteriorized. Three loops were created by means of ligatures, in the small intestine. The last of these loops extended to a point 15—20 cm proximal to the ileocaecal junction. Both the first and third loops, each of approximately 20 cm in length, were cannulated at their upper and lower ends. The intervening loop, which was only 5—8 cm long, was left intact and served to separate toxin-treated and control loops. The cannulae were secured with Mersilk ligatures and all blood vessels to the loops were left functional. The integrity of the blood supply was ascertained at the beginning and end of each experiment.

Salt solution prepared as in Pierce et al [9] was heated to 37°C in a heated coil water bath delivered at a rate of 1.0–1.7 ml/min. In all loops fluid flowed from the cephalic to the caudal end of the loop. Fluid was collected by downward drainage into 100 ml reservoirs. Perfusion of the loops was begun one hour after toxin administration and continued for some five hours. The volume delivered in any given hour, and the fluid output were measured. The percentage increase or decrease in fluid output were measured. The percentage increase or decrease in fluid output as compared to input was calculated.

One gram crude cholera toxin, Weyth 002 toxin, was dissolved in 5 ml salt solution used for perfusion and injected into the test loop. Control loops were inoculated with 5 ml salt solution. In each pair of experiments the positions of the test and control loops relative to the unperfused loop were altered in a cross-over design, so that an equal number of readings for test and control experiments for a particular region of the small intestine was obtained. The loops were held clamped off from the inflow and outflow cannulae for one hour.

Indomethacin was dissolved in 0.2 ml polyethylene glycol 200 and the volume for injection made up to 2.5 ml with 0.9% saline when administered intravenously. When the drug was locally applied into the lumen of the intestinal loops, the drug was dissolved in polyethylene glycol only. The doses of indomethacin used were 10 mg/kg, 50 mg/kg and 100 mg/kg intravenously and 600 µg/loop locally.

Acetylsalicylic acid either in the form of Alkaseltzer tablets or powdered acetylsalicylic acid were dissolved in alkaline salt solution and injected intravenously. The dose used was 25 mg/kg in all four experiments.

Intravenously administered drugs were given by slow injection over a two minute period some ten minutes prior to toxin challenge. Local indomethacin was delivered to the loop twenty minutes before toxin challenge or control solution was introduced into the loop.

RESULTS

The percentage change in fluid output compared with the amount of fluid delivered to each loop was calculated for each hour of perfusion. The results are given in Figures 1–6. Fig. 1 compares the responses of toxin-treated and control loops in animals receiving no anti-inflammatory drugs. Whereas toxin challenged loops showed a net secretion of fluid, the control loops showed a decrease in fluid output indicating that reabsorption of fluid had occurred throughout the five hours perfusion period. The greatest difference between the two loops is seen during the first hour of perfusion.

In all subsequent experiments fluid output from toxin-treated and control loops was measured after treatment with either indomethacin or aspirin. If these drugs were capable of antagonising the effects of cholera enterotoxin, then the secretory pattern of test and control loops should have been identical. The results are given in Figures 1–6.

Animals treated with intravenous indomethacin (Fig. 2-4).

In eight experiments indomethacin 10 mg/kg (5 animals) and 50 mg/kg (3 animals) injected intravenously had no significant effect on fluid output from toxin-treated or control loops. One animal received 100 mg/kg indomethacin. This higher dose caused a pronounced fall in arterial blood pressure but even in this experiment no significant effect on the action of cholera enterotoxin was observed.

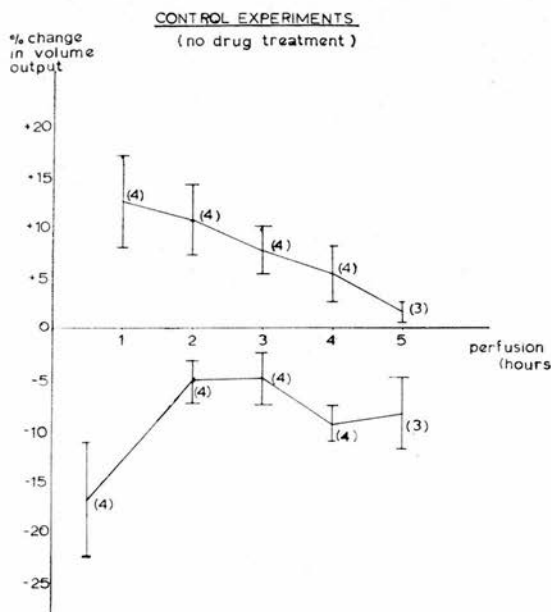


Fig. 1. Control experiments. The effect of cholera toxin on cat small intestine (upper graph) and the behaviour of salt solution treated loops (lower graph).

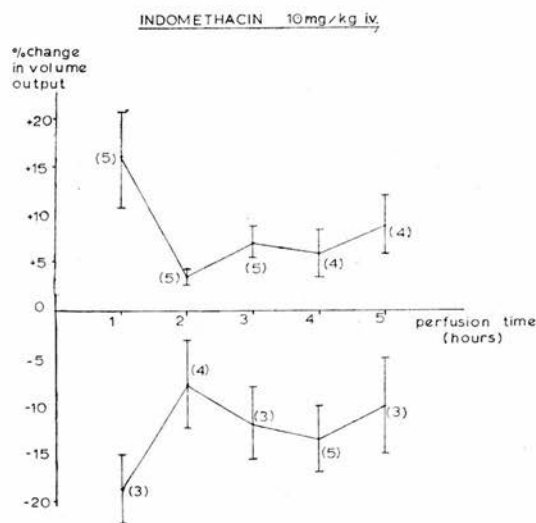


Fig. 2. The effect of indomethacin 10 mg/kg by intravenous injection on the responses of toxin-treated loops (upper graph) and salt-solution treated loops (lower graph) in cat small intestine.

Animals treated with local indomethacin (Fig. 5).

In three cats indomethacin (0.6 mg) was introduced into one toxin-treated loop. The other loop also received toxin but no anti-inflammatory drug. Again no significant effect on fluid output was detected following treatment with indomethacin.

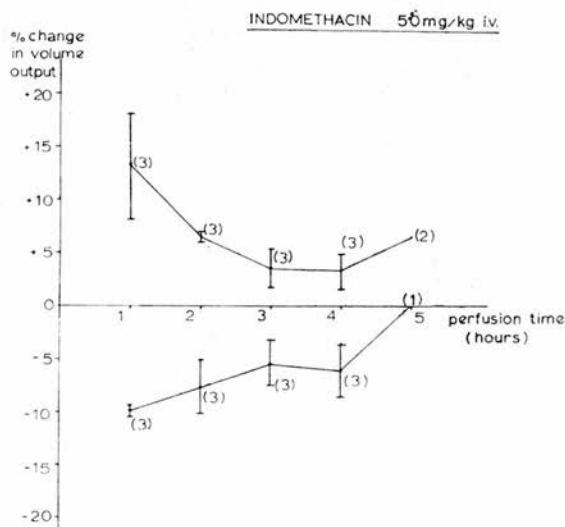


Fig. 3. The response of toxin-treated loops (upper graph) and salt solution treated loops following intravenous injection of indomethacin in a dose of 50 mg/kg.

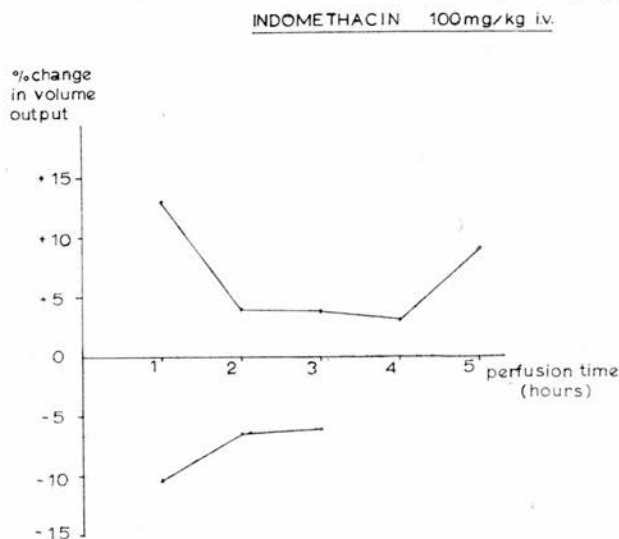


Fig. 4. The effect of 100 mg/kg indomethacin given by intravenous injection on cat small intestine exposed to cholera toxin (upper graph) and salt solution (lower graph).

Animals treated with aspirin intravenously

In cats (4 animals) aspirin in doses of 25 mg/kg also failed to modify fluid secretion in the toxin treated loops (Fig. 6).

Readings from loops which were seen to be dehydrated or to which the blood supply had become impaired were discarded. In some cases this resulted in only one reading for the five hour values, e.g. Fig. 3 control loop.

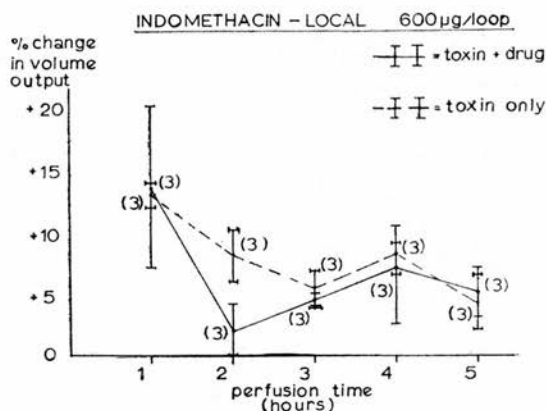


Fig. 5. The effect of locally administered indomethacin 600 μ g/loop on the response of cat terminal small intestine to challenge with cholera toxin (continuous-line graph) compared to toxin-challenge alone (broken-line graph).

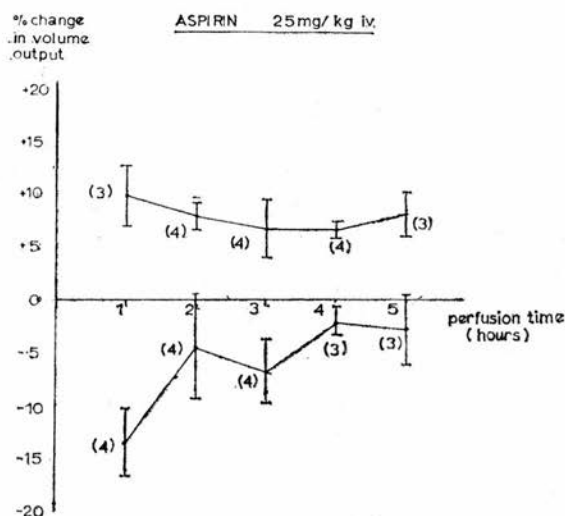


Fig. 6. The effect of 25 mg/kg aspirin administered intravenously on toxin-challenged loops (upper graph) and salt solution treated loops (lower graph) in cat small intestine.

Figs 1—6. The results of the control series of experiments are given on Figure 1. Those for the drug treated animals are summarized in Figures 2—6. Numbers in brackets indicate the number of observations made for each point on the graphs. The mean value for each point together with the standard deviation is plotted. The percentage change in fluid output compared to input is plotted against time for each series of experiments. In Figures 1—4 and Figure 6 the upper graph gives the results of the toxin treated loops and the lower graph those of the salt solution-treated loops. In Figure 5 the broken line gives the results of the toxin treated loops. Drug and toxin-treated loops results are depicted in the continuous line graph.

DISCUSSION

From these results it is concluded that the anti-inflammatory drugs, indomethacin and aspirin in the doses used were without significant effect on the secretion of fluid in the gut in the presence of cholera toxin.

The most significant period for monitoring the effects of potential anti-cholera drugs following toxin challenge is probably during the time of clamping off the gut and the first hour of perfusion. During the "incubation" interval the toxin-treated loop is observed to accumulate fluid whereas the untreated section of the intestine reabsorbs the fluid initially introduced into the lumen and does not produce any of its own accord. The secretion continues, in the case of the toxin-treated loop, for a period of several hours but effects from exteriorization and dehydration together with the trauma of continuous perfusion of fluid are not significant in the initial stages of the experiment. The intestine retains its capacity to reabsorb fluid, as is seen from the behaviour of the control loop. In order to exclude the possibility that lack of an adequate blood supply might contribute to the failure to observe any effects of the anti-inflammatory drugs, results from any loop found to have an impaired blood supply were rejected.

These results are in direct opposition to the results published by Fink and Katz [5]. These workers found that aspirin 25 mg/kg i.v. inhibited fluid secretion in the upper small intestine in the cat. A similar effect of anti-inflammatory drugs has been described by Jacoby and Marshall [7] in rat stomach and upper small intestine.

In both these experiments the investigators were studying a region of the alimentary tract specialized for secretory activity. The results presented in this paper, however, reflect the influence of drugs on that region of the small intestine associated mainly with reabsorption. It may well be that the discrepancy between these results and the results of the two other groups of workers is attributable to this basic physiological difference in epithelial cell type.

Bennett [1] has advocated the use of prostaglandin synthesis inhibitors in the treatment of human cholera, having suggested that prostaglandins might be involved as mediators of the toxin-induced diarrhoeas. Prostaglandins have been shown to cause diarrhoea in man. In 1969 Horton et al [6] and Misiewicz et al. [8] reported that oral PGE_1 caused massive diarrhoea of a watery nature and Cummings et al [3] described the production of diarrhoea in human subjects following intravenous infusion of prostaglandin $\text{F}_{2\alpha}$. It has also been suggested that the diarrhoeas associated with medullary carcinoma of the thyroid, carcinoid syndrome and ganglioneuroma may be attributable to the release of prostaglandins [10, 12].

Indomethacin and aspirin are prostaglandin synthesis inhibitors [4, 11]. Therefore, if prostaglandins were involved in the production of diarrhoea in cholera, one would have expected to see a change in the pattern of fluid

loss. There was no such alteration in these experiments. This, therefore, suggests that prostaglandins are not involved in the changes which occur in the terminal ileum induced by cholera toxin. It also indicates that, even if prostaglandins are implicated in the secretion of fluid in the upper small intestine, these anti-inflammatory agents will not necessarily prevent the symptoms of cholera since they do not reverse the inhibition of fluid reabsorption, nor the fluid secretion in more distal regions of the small intestine.

In a further paper evidence will be presented to support the belief that prostaglandins are not involved in the ileal responses to cholera toxin in the cat, and that doses of indomethacin and aspirin which failed *in vivo* to modify cholera toxin effects are capable of inhibiting *in vitro* synthesis of prostaglandins.

Bourne [2] suggested that the ability of aspirin and other prostaglandin antagonists to inhibit the actions of cholera toxin was due to the fact that other groups of workers were using suboptimal doses of toxin. The toxin used in the experiments presented in this paper is more potent on a weight basis than that used by Fink and Katz [5], (Toxin report sheet. Nat. Inst. Allergy and Infect. Dis. — Dr J. Seal). These results may, therefore, be in part attributable to this factor in addition to regional differences in the intestinal cell morphology.

The questions of doses of toxin and sensitivity of different areas of intestine to anti-inflammatory drugs require further investigation in order that these points may be clarified.

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DISCUSSION TO PAPER OF DR DLUGOLECKA

Bennet has suggested that cholera toxin stimulates the release or synthesis of prostaglandins, which in turn stimulate the c-AMP synthesis and diarrhea results. Several authors have used PGE inhibitors and demonstrated some inhibition of intestinal fluid secretion. These findings would suggest the major role of PG in cholera induced diarrhea. The findings of Dr Dlugolecka speak against this interpretation.

According to our findings concerning both cholera toxin and *E. coli* enterotoxin (which also stimulates adenylcyclase activity) actions and from data in the literature it seems not to be probable that the main action of both toxins is mediated by PGE only.

All adenylate cyclase preparations stimulated by cholera toxin keep the sensitivity to hormone stimulation. However the absence of substantial increase by hormone stimulation in cholera toxin treated tissues suggest that the activation of the enzyme by toxins and hormones is of different nature. It seems that both cholera toxin and *E. coli* enterotoxin

enlarge that part of the regulatory unit of adenylate cyclase, which is responsible for basal activity and not the unit sensitive to hormone stimulation. However this part might be in vivo stimulated by liberated PG due to other actions of cholera toxin and *E. coli* enterotoxin.

This view is supported by differences in the action of cholera toxin and prostaglandins (time, course, intensity, effect from serosal and mucosal site, possibility to reduce the effect by washing and stimulation in brush cell preparation).

M. J. Długolecka

BRAK MODYFIKOWANIA ODPOWIEDZI JELITA CIENKIEGO KOTA NA ENTEROTOKSYNĘ CHOLERY PRZEZ INDOMETACYNĘ

Streszczenie

Badano wpływ dwóch inhibitorów syntezy prostaglandyn i związków przeciwzapalnych u 21 kotów, których końcowa część jelita cienkiego była poddana działaniu toksyny cholery.

Ani indometacyna (10, 50 lub 100 mg/kg i.v., bądź 600 µg na pętlę lokalnie), ani aspiryna (25 mg/kg iv) nie znosiły odpowiedzi jelita na toksynę. Wydaje się, że prostaglandyny nie są prawdopodobnie mediatorami utraty płynu obserwowanej w końcowym jelicie cienkim.

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